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<b>(54) Title:</b> GENETIC TRANSFORMATION USING A PARP INHIBITOR  <b>(57) Abstract</b>  The invention concerns a process for producing transgenic eucaryotic cells, particularly plants, which comprises: contacting a culture of untransformed cells with an inhibitor of poly-(ADP-ribose) polymerase for a period of time sufficient to reduce the response of the cultured cells to stress and to reduce their metabolism. The untransformed cells are then contacted with foreign DNA comprising at least one gene of interest under conditions in which the foreign DNA is taken up by the untransformed cells and the gene of interest is stably integrated in the nuclear genome of the untransformed cells to produce the transgenic cells. Optionally, the transgenic cells are recovered from the culture. Preferably, the inhibitor is niacinamide, preferably at a concentration of about 200 mg/l to 500 mg/l and the untransformed cells are cultured in a medium containing the inhibitor for a period of time of approximately 3 to 14 days prior to the contacting with the foreign DNA. The invention also relates to a plant having in the nuclear genome of its cells foreign DNA integrated only in the regions of the nuclear genome that are transcriptionally active in cells of the plant when the cells are treated with an effective amount of a PARP inhibitor for a period of time sufficient to reduce cell metabolism to a state where gene expression is essentially limited to genes expressed irrespective of the differentiated or physiological condition of the cell.		

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## GENETIC TRANSFORMATION USING A PARP INHIBITOR

This invention is related to tissue culture of eucaryotic cells and improved techniques to obtain genetically transformed eucaryotic cells and organisms, such as transgenic plant cells or plants, by lowering the stress reaction of cultured eucaryotic cells prior to contacting the cells with foreign DNA, particularly by specific inhibition of poly-(ADP-ribose) polymerase.

Background to the invention

Over the years many techniques for the genetic transformation of higher organisms (animals and plants) have been developed. In these techniques it is the ultimate goal to obtain a transgenic organism, e.g. a plant, in which all cells contain a foreign DNA comprising a gene of interest (the so-called transgene) stably integrated in their genome, particularly their nuclear genome.

Transformation is a complex process which always involves the contacting of starting cells with a DNA, usually a DNA comprising foreign gene(s) of interest. The contacting of the cells with the DNA is carried out under conditions that promote the uptake of the DNA by the cells and the integration of the DNA, including the gene(s) of interest into the genome of the cell.

Starting cells for transformation are usually cells that have been cultured in vitro for some time. After contacting the cells with the DNA, the transformed cells generally need to be cultured in vitro for a certain period in order to separate the transformed cells from the non-transformed cells and, in the case of plants, to regenerate transformed plants from the transformed cells. Indeed, complete plants can be regenerated from individual transformed cells thus ensuring that all cells of the regenerated plant will contain the transgene.

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In many plants, genetic transformation can be achieved by using the natural capacity of certain Agrobacterium strains to introduce a part of their Ti-plasmid, i.e. the T-DNA, into plant cells and to integrate this T-DNA into the nuclear genome of the cells. It was found that the part of the Ti-plasmid that is transferred and integrated is delineated by specific DNA sequences, the so-called left and right T-DNA border sequences and that the natural T-DNA sequences between these border sequences can be replaced by foreign DNA (European Patent Publication "EP" 116718; Deblaere et al, 1987 Meth.Enzymol. 153:277-293).

Certain plant species have proven to be recalcitrant to Agrobacterium mediated transformation and in these species, as well as in animals, genetic transformation has been achieved by means of direct gene transfer by which DNA is inserted into the cells by physical and/or chemical means, such as by electroporation, by treatment of the cells with polyethyleneglycol (PEG), by bombardment of the cells with DNA-coated microprojectiles, etc. (WO 92/09696; Potrykus et al, 1991, Annu.Rev.Plant Physiol.Plant Mol.Biol. 42:205-225).

Genetic transformation of eucaryotic cells is generally a random event; i.e. the transgene is integrated in the genome at random positions. Often several copies (or parts of copies) of the transforming DNA are integrated in a single position, and/or at different positions, resulting in a transformed cell containing multiple copies of the transgene.

The expression of the transgene is known to be influenced by its position in the genome. For instance, a foreign DNA, when introduced in a plant cell appears to integrate randomly in the plant genome. Examination of independently transformed plants has shown a high degree of variability (up to 100-fold) in the expression level of the introduced gene. Several studies have shown no correlation between this "between-transformant variability" and the copy

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number of the introduced DNA at a given locus. It has been suggested that some of the variability in expression of introduced genes in transgenic plants is a consequence of "position effects" caused by influences of adjacent plant genomic DNA. Other factors that could contribute to the variability in expression are physiological variability of the plant material, differences in the number of independent T-DNA loci in different transformants or the inhibitory effects of certain T-DNA structures on gene expression. Between-transformant variability in expression has been observed for the majority of introduced genes in transgenic plants. The variability in expression of many introduced genes in independent transgenic plants necessitates large numbers of transgenic plants to be assayed to accurately quantitate the expression of the gene. It would be of great importance if the amount of between-transformant variability could be reduced (Dean et al, 1988, NAR 16:9267-9283).

If the transgene is under the control of a tissue-specific promoter, with the expectation that it will be expressed in selected tissues of the transformed organisms, the position effects can lead, at least in some transformants, to loss of specificity of the promoter and expression of the transgene in undesired tissues, e.g. in tissue cultured in vitro.

Factors that are known to influence the efficiency and quality of the genetic transformation process are the method of DNA delivery, specific tissue culture conditions, the physiological and metabolic state of the target cells etc. Direct gene transfer methods for instance are generally known to result in transformed organisms with a high copy number of the transgene.

Many of these factors are not under the control of man.

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### Summary of the Invention

This invention provides a process for producing transgenic eucaryotic cells, particularly plant cells. The process comprises contacting a culture of untransformed cells with an

5 inhibitor of poly-(ADP-ribose) for a period of time sufficient to reduce the response of the cultured cells to stress and to reduce the metabolism of the cultured cells, particularly to reduce the electron flow in the mitochondrial electron transport chain. The untransformed cells are then contacted with foreign DNA comprising at least one gene of interest under conditions in which  
10 the foreign DNA is taken up by the untransformed cells and the gene of interest is stably integrated in the nuclear genome of the untransformed cells to produce the transgenic cells which are recovered from the culture.

The process may preferably comprise contacting untransformed eucaryotic  
15 (e.g.) cells with foreign DNA comprising at least one gene of interest under conditions in which the foreign DNA is taken up by the untransformed cells and the gene of interest is stably integrated in the nuclear genome of the untransformed cells to produce the transgenic cells. The untransformed cells are cultured in vitro in a culture medium containing an inhibitor of poly-(ADP-  
20 ribose) polymerase, preferably niacinamide, preferably for at least 2 to 3 days, particularly for at least 4 days (e.g. 4-5 days), before the contacting of the untransformed cells with the foreign DNA. The inhibitor can in addition also be applied to cultured cells that are being contacted or that have been contacted with the foreign DNA.

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### Description of the Invention

The present invention is based on the observations that poly-(ADP-ribose) polymerase (PARP) is an enzyme that is involved in regulating the general metabolic state of an eucaryotic cell and that inhibition of this enzyme can be

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used to influence the metabolic state of cells which are targeted for transformation (or which are being transformed) to increase the efficiency and/or quality of transformation.

- 5 In mammals, PARP is a monomeric nuclear Zn-finger protein of about 116 kD that is closely associated with nuclear DNA, particularly with actively transcribed euchromatic regions (Shah et al, 1995, *Anal.Biochem.* 227:1-13). The protein is normally an inactive enzyme but is known to be activated by nicked or otherwise damaged DNA. Active PARP transfers the ADP-ribose moiety of NAD<sup>+</sup> to various nuclear proteins to synthesize a polymer of ADP-ribose bound to these proteins which include PARP itself, polymerases, histones, endonuclease etc. The proteins on which such a ADP-ribose polymer is synthesized become biologically inactive (de Murcia et al, 1994, *TIBS* 19:172-176; Cleaver et al, 1991, *Mutation Res.* 257:1-18).
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- 15 The biological function of PARP is largely unknown but the enzyme has been implicated in :
- enhancement of DNA repair (Satoh et al, 1992, *Nature* 356:356-358; Satoh et al, 1993, *J.Biol.Chem.* 268:5480-5487),
  - recombination events : in general inhibition of PARP is observed to inhibit illegitimate recombination and to increase intrachromosomal recombination but it does apparently not affect extrachromosomal recombination (Farzaneh et al, 1988, *NAR* 16:11319-11326; Waldman and Waldman, 1990, *NAR* 18:5981-5988; Waldman and Waldman, 1991, *NAR* 19:5943-5947),
  - 20
  - 25 - regulation of gene expression : inhibition of PARP is observed to decrease gene expression (Girod et al, 1991, *Plant Cell, Tissue and Organ Culture* 25:1-12);
  - reducing the amount of available NAD<sup>+</sup> (and by consequence its precursor ATP) : this results in a general slowing down of cell metabolism (Lazebnik

et al, 1994, 371:346-347; Gaal et al, 1987, TIBS 12:129-130; Cleaver et al, supra)

It is known that PARP can be efficiently inhibited by a number of compounds  
5 (Durkacz et al, 1980, Nature 283:593-596; Sims et al, 1982, Biochemistry  
21:1813-1821). Examples of such compounds are certain pyridine analogs  
such as nicotinamide analogues, including niacinamide, picolinamide, and 5-  
methyl nicotinamide; purine analogs like methylxanthines; thymidine;  
pyrazinamide analogs and many aromatic amides such as many benzamide  
10 analogs including benzamide, 3-methoxybenzamide and 3-aminobenzamide.  
For the purpose of this invention a PARP inhibitor is generally understood as  
any specific inhibitor of poly-(ADP-ribose) polymerase which can be taken up  
by a eucaryotic cell, particularly a plant cell, and which has a inhibition constant  
( $K_i$ ) which is lower than  $1 \times 10^{-5}$ , particularly lower than  $1 \times 10^{-6}$ . Generally it is  
15 desired that the PARP inhibitor used with this invention be a compound which  
in human lymphocytes, cultured in medium containing the inhibitor at a  
concentration of 2 mM, results in a 80-90 % inhibition of PARP. (Sims et al,  
supra). Generally it is also preferred that cells cultured in medium containing  
the PARP inhibitor retain their capacity of DNA repair.

20 Particularly preferred PARP inhibitors are those listed above and especially  
niacinamide (nicotinamide), picolinamide, 5-methylnicotinamide, 2-  
aminobenzamide, pyrazinamide, theobromine and theophylline. Particularly  
niacinamide is believed to be a useful inhibitor for the purpose of this invention.

25 Basically the present invention provides a modification of existing procedures  
for the genetic transformation of eucaryotic cells, particularly plant cells, by  
including in the medium in which such cells are cultured a PARP inhibitor such  
as niacinamide, for a defined period of time. In particular the PARP inhibitor is  
added to the culture medium at least 1 day prior to the moment (the "contacting



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time") at which the cells are contacted with foreign DNA comprising one or more genes of interest. However, depending on the purpose, the PARP inhibitor may also be added to the culture medium during and/or after the contacting time or even solely after the contacting time.

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In one aspect of this invention treatment of cultured cells, tissues or organs with PARP inhibitors may be used to increase the quality of transformation as measured by the copy number of the transgene and by variation in transgene expression (quality and quantity) in the transformed cells and in organisms obtained from the transformed cells.

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In many conventional procedures for genetic transformation of eucaryotic cells, particularly plant cells, cultured cells, tissues or organs will be used as starting material and cells in such cultures will be contacted with foreign DNA comprising at least one gene of interest (i.e. the transgene) under conditions that will promote the uptake of the foreign DNA in the cells and the ultimate integration of the foreign DNA into the genome of the cells.

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In one embodiment of the invention it is preferred that a PARP inhibitor is added to the culture medium for a period of at least 2-3 days, preferably at least about 3 days, prior to contacting the cells with the foreign DNA. The exact period in which the cultured cells are incubated in PARP inhibitor containing medium is believed not to be critical but should probably not exceed 4 weeks. It appears that 2-14 days, particularly 3-10 days, is an optimal period and best results were obtained with an incubation period of approximately 4 to 5 days prior to the contacting time. Generally it is believed that 4 days is a useful period for the PARP inhibitor to be added to the culture medium prior to the contacting time.

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The concentration of the PARP inhibitor in the medium is also believed to have an effect on the inhibition of PARP, which varies depending on the nature of the cells (species, tissue explant, general culture conditions, etc.) However,

within certain concentration ranges, the effect is minimal, especially when the cultured cells are not incubated for longer than 14 days. The optimal concentration range of PARP inhibitor in the medium may vary depending on the species from which the tissue, cell or cell culture is derived, but 250 mg/l (about 2 mM) is believed to be a suitable concentration for many purposes (e.g. for use with material derived from wheat). However, when nicotinamide is used in combination with plant material derived from rice, the concentration of nicotinamide should preferably be between 500 mg/l (about 4 mM) and 1000 mg/l (approx. 8 mM). On the other hand, when nicotinamide is used in combination with plant material derived from corn, the concentration of nicotinamide should preferably be 100 mg/l. Likewise, a concentration of 100 mg/l is already effective for wheat-derived plant material, but higher concentrations may be used. The optimal concentration will depend on the nature of the specific PARP inhibitor used, particularly on its strength of inhibition (as measured by its  $K_i$  and/or by its percentage inhibition of PARP under standard conditions - Sims et al, supra). It was found for instance that the optimal concentration for nicotinamide is approximately 250 mg/l (i.e. about 2 mM) but it is believed that concentrations up to 1000 mg/l (approx. 8 mM) and as low as 150 mg/l (approx. 1.25 mM), even as low as 100mg/l can be used to good effect. Preferably the nicotinamide concentration should be between 200 and 300 mg/l, i.e. between approximately 1.5 mM and 2.5 mM. In similar conditions, the optimal concentration for more potent PARP inhibitors such as 3-methoxybenzamide is about 0.5 mM, but it is believed that concentrations up to 2 mM and as low as 0.1 mM can be used to good effect. Similar concentrations apply to other PARP inhibitors.

If incubation times of longer than 14 days are used it is believed that the PARP inhibitor concentration should be reduced below 2 mM (e.g. between 0.5 mM and 1.5 mM and particularly approximately 0.8 mM).

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For other PARP inhibitors optimal concentrations can be easily established by experimentation in accordance with this invention.

During transformation it is not known whether the integration of the DNA into the genome of the cell occurs immediately after uptake of DNA by the cell. It may very well be that the foreign DNA exists as free DNA within the cell for a certain period after the contacting time. Therefore cultured cells may be further incubated in medium containing a PARP inhibitor during and, for a limited period of time after, contacting the cells with the foreign DNA. Again the length of the incubation period is not critical but is preferably 2-10 days, particularly approximately 4 days. It is preferred that the inhibitor concentration of the PARP inhibitor in the culture medium after the contacting time should be below 2 mM, between 0.8 and 1 mM. If the cells that are to be transformed are not obtained from a cell or tissue culture (e.g. when intact tissue of an organism is contacted directly with DNA, as for example described in WO 92/09696) the PARP inhibitor may still be applied to the target cells prior to the contacting time but the addition of the PARP inhibitor to the culture of the transformed cells during or after the contacting time is preferred.

As indicated above, PARP inhibitor treatment of cultured cells for at least 2-3 days increases the quality of transformation. Indeed the number of copies of the foreign DNA is expected to be generally lower and variation in expression profile (level - i.e. the quantity - of expression as well as spatial and time distribution - i.e. the quality - of expression in the transgenic organism) of the gene(s) of interest in the foreign DNA, due to position effects, is decreased. However, at least in this aspect of the invention, the efficiency of transformation can be decreased. The efficiency of transformation as used herein can be measured by the number of transformed cells (or transgenic organisms grown from individual transformed cells) that are recovered under standard experimental conditions (i.e. standardized or normalized with respect to amount

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of cells contacted with foreign DNA, amount of delivered DNA, type and conditions of DNA delivery, general culture conditions etc.).

Therefore it is preferred that the invention is used with transformation procedures that already have a high efficiency, such as Agrobacterium mediated transformation of dicots and direct gene transfer in monocots, particularly cereals (e.g. electroporation or particle bombardment of compact embryogenic callus in cereals - see WO 92/09696). Indeed these transformation procedures are generally highly efficient but the quality of transformation is generally poor. Position effects are large and, especially with direct gene transfer, the copy number of the transgene is often exceptionally high making analysis and selection of optimal transformants, as well as further breeding with the transformants, difficult.

In another aspect of this invention treatment of cultured plant cells for a short period of time (i.e. 1 day to maximally 2 days) prior to, or after contacting the cells with DNA may be used to increase the efficiency of Agrobacterium mediated transformation of plant species, such as many monocots, particularly the major cereals such as wheat and corn, for which this method is generally inefficient. It is believed that treatment of cultured plant cells during the contacting time may result in a lower transformation efficiency, and might therefore not be suitable for this aspect of this invention. Likewise, it is believed that for the purpose of this aspect of the invention, the optimal treatment with a PARP inhibitor is 1 day to maximally 2 days prior to the contacting time, or alternatively 1 to maximally 2 days after the contacting time. In this embodiment of the invention the contacting of the plant cells with the DNA should of course be understood as contacting the cells with an appropriate Agrobacterium strain harboring an artificial T-DNA containing the foreign DNA with the gene(s) of interest. In this embodiment of the invention the quality of transformation is expected not to be affected but this is generally deemed to be

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of lesser importance since Agrobacterium mediated transformation, being a biological process, already results in a generally low copy number of the transgene in the transformed plant cells.

5 In accordance with this invention the addition of PARP inhibitors, such as niacinamide, to the culture medium of eucaryotic cells, can be used in combination with any known transformation procedure that requires cells, tissues or organs cultured in vitro as starting cells to be contacted with foreign DNA. The process of this invention is thus generally identical to existing  
10 conventional transformation methods except for the fact that at some times during the tissue culture of the cells, a PARP inhibitor is added to the culture medium.

The cell of a plant, particularly a plant capable of being infected with Agrobacterium such as most dicotyledonous plants (e.g. Brassica napus) and  
15 some monocotyledonous plants, can be transformed using a vector that is a disarmed Ti-plasmid containing the gene(s) of interest and carried by Agrobacterium. This transformation can be carried out using conventional procedures (EP 0,116,718; Deblaere et al, supra; Chang et al, 1994, The Plant Journal 5:551-558). Preferred Ti-plasmid vectors contain the foreign DNA  
20 between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in EP 0,233,247), pollen mediated transformation (as described, for example, in EP 0,270,356, PCT patent publication "WO" 85/01856, and US patent 4,684,611), plant RNA virus-  
25 mediated transformation (as described, for example, in EP 0,067,553 and US patent 4,407,956) and liposome-mediated transformation (as described, for example, in US patent 4,536,475). Cells of monocotyledonous plants such as the major cereals including corn, rice, wheat, barley, and rye, can be

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transformed (e.g. by electroporation) using wounded or enzyme-degraded intact tissues capable of forming compact embryogenic callus (such as immature embryos in corn), or the embryogenic callus (such as type I callus in corn) obtained thereof, as described in WO 92/09696. In case the plant to be transformed is corn, other recently developed methods can also be used such as, for example, the method described for certain lines of corn by Fromm et al., 1990, Bio/Technology 8:833; Gordon-Kamm et al., 1990, Bio/Technology 2:603 and Gould et al., 1991, Plant Physiol. 95:426. In case the plant to be transformed is rice, recently developed methods can also be used such as, for example, the method described for certain lines of rice by Shimamoto et al., 1989, Nature 338:274; Datta et al., 1990, Bio/Technology 8:736; and Hayashimoto et al., 1990, Plant Physiol. 93:857; Hiei et al., 1994, The Plant Journal 6:271-282).

The transformed cell can be regenerated into a mature plant and the resulting transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the gene(s) of interest in other varieties of the same related plant species. Seeds obtained from the transformed plants contain the chimeric gene(s) of this invention as a stable genomic insert. Thus the gene(s) of interest when introduced into a particular line of a plant species can always be introduced into any other line by backcrossing.

In animals pluripotent embryonic or somatic stem cells can be used as target for transformation (Capecchi et al, 1989, TIG:5:70-76).

The transformed cells and organisms of any plant or animal species, produced by the process of this invention, contain the foreign DNA as a stable insert in their genome, particularly in regions of the genome that remain transcriptionally active in the untransformed cells that have been exposed to a PARP inhibitor in

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accordance with this invention. As described above it is believed that in cells treated with a PARP inhibitor for at least 3 days, particularly for at least 4 days, only a limited number of genomic regions will remain transcriptionally active. In this regard the transformed cells, obtained with this process of the invention, will be characterized by having the foreign DNA integrated in a limited number of genomic regions. That the transformed cell or organism was obtained by this process of the invention can thus be easily ascertained by 1) culturing transformed cells or tissues under conditions that are similar as those in which the untransformed cells or tissues were grown or incubated prior to the integration of the foreign DNA in the genome (i.e. incubating in medium containing 250 mg/l niacinamide for 4-5 days prior to the contacting time), and 2) monitoring the expression of at least one transgene in the foreign DNA that is expected to be expressed under normal tissue culture conditions (i.e. a selectable marker gene under the control of a promoter that directs expression in tissue culture). Under the above conditions the transformed cells or tissues of this invention express the relevant transgene in the tissue culture at essentially the same levels whether or not a PARP inhibitor is present in the culture medium. It is thus expected that, for instance after 4-5 days of culturing of the transformed cells in medium containing the PARP inhibitor, mRNA levels are not significantly decreased, i.e. do not become lower than 75%, preferably not become lower than 90%, when compared to the mRNA levels observed in cells cultured in medium not containing the inhibitor. Indeed, if the relevant transgene is integrated in other regions of the genome (i.e. in regions that are normally not transcriptionally active in cells treated with PARP inhibitor according to this embodiment of the invention), the expression of the relevant transgene is considerably reduced after incubation of the cells in medium containing the PARP inhibitor for at least 3 days, e.g. 4-5 days (i.e. mRNA levels will drop below 75%, particularly below 50%, more particularly below

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30%) when compared to the mRNA levels observed in cells cultured in medium not containing the inhibitor).

5 The method of the present invention can in principle be used to transform eucaryotic cells with any foreign DNA. Generally the foreign DNA comprises at least one gene of interest comprising 1) a promoter region with a promoter capable of directing transcription of DNA into a RNA in cells of the eucaryotic, e.g. plant, species that is to be transformed and 2) a coding region coding for a RNA or protein. Most often the gene of interest will also comprise 3) a 3'  
10 untranslated region of a eucaryotic gene containing a polyadenylation signal.

The promoter can be selected to direct expression in selected tissues of the eucaryotic organism. Such a tissue-selective promoter is not expected to direct expression in other non-selected tissues. For instance promoters are known that direct expression selectively in stamen tissues of a plant and such  
15 promoters have been used to produce male sterile plants and other plants useful for producing hybrids (EP 344029; EP 412911; WO 9213956; WO 9213957; Mariani et al, 1990, Nature 347:737-741; Mariani et al, 1992, Nature 357:384-387).

It is believed that the method of the present invention is particularly useful to  
20 transform eucaryotic cells with at least one gene of interest comprising a tissue-selective promoter, such as a stamen selective promoter, especially if expression of the gene of interest in the organism, such as a plant, outside the selected tissues (where the tissue-selective promoter is active, i.e. directs expression) is undesired for example because the gene product (for instance a  
25 protein such as a ribonuclease, e.g. barnase) is capable of killing or disabling the cells in which they are produced. In such cases expression of the gene of interest in tissue culture, or in non-selected tissues of the organisms can negatively affect the quality as well as the apparent efficiency of transformation. When the method of this invention is used, the overall efficiency of



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transformation may be reduced but the average quality of transformation is expected to be significantly improved because of lower copy number of the gene of interest in the genome of the transformed cells and because of reduced position effects i.e. the general integration of the gene of interest in the genomes at locations that minimally affect the transcriptional properties of the promoter of the transgene.

The foreign DNA used in the method of this invention generally also comprises a selectable marker gene the expression of which allows the selection of transformed cells (or organisms) from non-transformed cells (or organisms).

Such selectable marker gene generally encodes a protein that confers to the cell resistance to an antibiotic or other chemical compound that is normally toxic for the cells. In plants the selectable marker gene may thus also encode a protein that confers resistance to a herbicide, such as a herbicide comprising a glutamine synthetase inhibitor (e.g. phosphinothricin) as an active ingredient.

An example of such genes are genes encoding phosphinothricin acetyl transferase such as the sfr or sfrv genes (EP 242236; EP 242246; De Block et al, 1987 EMBO J 6:2513-2518).

The inventors also found that the initial reaction of cells, particularly cells contacted with PARP inhibitors, is a stress reaction which enhances free radical production by the cell. However, this stress only lasts for a limited period of time after which further contact with the PARP inhibitor causes a decrease in cell metabolism, particularly a decrease in electron flow in the mitochondrial electron transport chain. Therefore, the invention also relates to a new method to assess the agronomical fitness of a population of transformed plants to determine in which lines the plants have a foreign DNA integrated in their genomes in a way that agronomical performance is not or substantially not affected. The assay is based on comparative reaction of transgenic cells and corresponding untransformed controls to stress conditions.

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The method comprises exposing the transgenic cells to stress conditions which induce the production of free radicals in the tissues or the cells, measuring the amount of free radicals produced in the transgenic cells with the amount of free radicals produced in control cells exposed to similar stress conditions. Preferably the cells of the transgenic organism to be assayed are exposed to stress conditions by being treated with a substance which induces increasing osmotic and/ or salt stress on the cells.

The properties of PARP inhibitors, such as niacinamide, to enhance free radical production in cells incubated with the inhibitor for not longer than 2 days, preferably not longer than 1 day, can be used to assay the (relative) fitness of a population of transgenic eucaryotic organisms, particularly plants.

The term fitness used herein is intended to designate the agronomical performance of a population of plants, as measured for instance by its yield (e.g. its seed yield) as compared to a given reference population. Agronomical performance is generally thought to be correlated with the general resistance of the plants to a range of stress conditions which are likely to be encountered in the field locations where the plants are normally grown. For any population of transformed plants (i.e. a transgenic line) the relevant reference population is a population of untransformed plants of the same variety.

It is known that in transformed plants and other organisms transgene expression may be qualitatively and quantitatively influenced by the genomic domain in which the transgene(s) are integrated, that undesired transgene expression may interfere with cell metabolism (e.g. when the transgene encodes a cytotoxic protein), that mutations may be induced in the transformed organism either by somaclonal variation or by insertional inactivation of

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endogenous genes by the transgene(s), or that expression of endogenous genes may be deregulated by sequences in the foreign DNA. As a consequence many transformed lines may not be agronomically useful.

5 The assay of this invention will for example allow to identify a line (i.e. a group of genetically similar plants) of transformed plants that have the transgene(s) integrated in regions that minimally affect the fitness of the plants, thus avoiding the extensive laboratory, greenhouse and/or field evaluations which are normally required to identify the transformants with the best agronomical properties.

10 The assay in accordance with this invention essentially comprises the incubation of cells or tissues of transformed plants of a particular transgenic line (e.g. callus, hypocotyl explants, shoots, leaf disks, whole leaves etc.) preferably with a PARP inhibitor (although for some plant species this is not  
15 necessary) under a range of conditions which induce the production of a different amount of free radicals in the tissues. An incubation time of approximately one day is normally sufficient to generate the desired amount of free radicals. Appropriate controls, i.e. untransformed tissues obtained from untransformed plants at the same developmental stage and grown in the same  
20 conditions as the transformed plant from which the transformed tissue was obtained, are subjected to the same treatment. Preferably the untransformed line is identical to the transgenic line except for the presence of the transgene(s).

25 For each plant line (control or transformant) it is preferred that a number of plants is assayed.

Useful conditions for the incubation of the untransformed and transformed tissues are those which induce increasing osmotic and salt stress in the incubated cells or tissues. For example a series of buffers with different salt

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concentrations containing a PARP inhibitor can be made. A useful buffer series is a K-phosphate buffer containing 2% sucrose and 250 mg/l niacinamide in which the K-phosphate concentration is increased from anywhere between 10 to 80 mM (e.g. in steps of 5 mM, i.e. 10, 20, 25, 30, 35, 40, 45, 50, 55, 60 mM). The K-phosphate concentrations will induce mild but increasing salt and osmotic stress in plant cells. The niacinamide in the medium further enhances radical production and stress on the plant cells. The range of K-phosphate concentrations used will depend on the natural sensitivity of the plant species (or if desired the plant line) to the salt and osmotic stress. In sensitive plant species, which will not tolerate high salt stress, the maximum K-phosphate concentration may for instance be 50 mM, in less sensitive species this maximum K-phosphate concentration can be increased up to 70 or 80 mM or even higher. For each plant species the minimum and particularly the maximum salt (e.g. K-phosphate) concentration can be determined experimentally for an untransformed line - the only requirement is that at all concentrations used the plant tissue remains viable. Although the addition of a PARP inhibitor to the medium, such as niacinamide, is preferred it is not required for assaying plant species that are very sensitive to salt and/or osmotic stress.

After the one day incubation the capacity of the transformed and control tissues to reduce 2,3,5-triphenyltetrazolium chloride (TTC) is measured e.g. by the following procedure which is modified from Towill and Mazur (supra):

- incubate the tissues for 1 to 4 hours in K-phosphate buffer (pH 7.4) containing 10 mM TTC and 0.1 % Tween20. As a control similar plant material is incubated in the same buffer without TTC.
- extraction of reduced TTC (e.g. freezing at -70°C followed by thawing at 40°C and shaking the plant material in ethanol for 45-60 minutes)

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- spectrophotometric quantification of reduced TTC at 485 nm (optical density OD<sub>485</sub>; for chlorophyll poor plant material) or 545 nm (OD<sub>545</sub>; for chlorophyll rich plant material). The O.D. of the control extract is subtracted from the OD of the TTC-reacted extracts. In the above conditions 0.1 mM reduced TTC corresponds to an OD<sub>485</sub> of 0.214 or OD<sub>545</sub> of 1.025 (light path 1 cm).
- the reducing capacity of the transformed plant line is compared to that of the control line.

The amount of reduced TTC is determined by the intensity of the cytochromal and alternative respiratory pathways and the radical concentration in the tissues which, in turn are determined by the presence of mutations, the expression of genes affecting the metabolic activity of the plant cells, the developmental stage and the reaction of the tissue to external factors, such as stress factors.

The TTC reducing capacity (as for instance measured by the O.D. at 485 nm) for tissues incubated at high salt concentration (TTC-high) is expressed as the percentage of the TTC reducing capacity of the tissues incubated at a low salt concentration (TTC-low); in other words a TTC-ratio value is calculated as follows:

$$\text{TTC-ratio} = \text{TTC/high} \cdot 100 / \text{TTC/low}.$$

The value of TTC-ratio is a measure of the fitness of a plant line as compared to a control line.

The determination of TTC-low and TTC-high will depend on the sensitivity of the plant species to the applied salt stress. Usually TTC-low will correspond to a salt concentration between 10 and 25 mM K-phosphate, e.g. at 20 mM while TTC-high will correspond to a salt concentration between 50 and 80 mM K-phosphate. The only requirement is that TTC-high should be significantly lower than TTC-low; preferably TTC-high should be lower than 50% of TTC-low, particularly lower than 30% of TTC-low. For instance for Brassica napus, TTC-

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low and TTC-high can be typically obtained from tissues incubated at respectively 20mM and 60 mM K-phosphate buffer containing 250 mg/l niacinamide. TTC-high and TTC-low, for both the transformed and untransformed line, will usually be an average obtained from several measurements taken on a number of tissue explants from a number of plants of each line. For instance for each line of Brassica napus about 32 leaf discs (diameter 1 cm) from 8 different plants (i.e. about four leaf discs per plant) can be assayed to determine 32 TTC-high and 32 TTC-low values which are averaged to obtain the TTC-high and TTC-low values used for the calculation of TTC-ratio. Other examples of sample sizes which have been used are 35 shoots from Arabidopsis thaliana, or 150 hypocotyl explants derived from about 25 seedlings of Brassica napus.

Transformed lines with a value of TTC-ratio which does not deviate more than 20%, preferably not more than 10% of the TTC-ratio value of the control line are selected. These lines are likely to have the transgene(s) integrated in regions that minimally affect the fitness of the plants.

It is clear that additional information considering the fitness of the plant material studied can be obtained by comparing the TTC-reducing capacity of the plant material in absence of a PARP-inhibitor with the TTC-reducing capacity of the plant material in the presence of a PARP-inhibitor for each experimental point of the buffer series mentioned above.

While the TTC-reduction assay is especially suitable for the identification of transgenic plants, where transgenes are integrated in regions that minimally affect the fitness of the plants, this test can also be successfully applied to discriminate mutant plants, cells or cell lines from the wild-types.

The TTC-reducing assay can further be used in a modified way to determine the quality and the fitness of plant material, for example plant material to be used in transformation experiments (i.e. whether particular plant material, e.g. explants, is suitable as starting material). To this end the TTC-reducing assay can be adapted for example in the following way:

1. A sample of the plant material to be tested for its suitability for transformation, is incubated for one day in plant culture medium or a buffer containing 2% sucrose and a K-phosphate concentration ranging between 10 and 80 mM, typically around 25 mM, to which a suitable amount of a PARP inhibitor, such as niacinamide has been added. For niacinamide, a preferred concentration to be used is 250 mg/L, although concentrations as low as 100 mg/L and as high as 1000 mg/L may be used. A comparable control sample of the same plant material is incubated under similar conditions without PARP inhibitor.
2. After one day of incubation the capacity of the plant material incubated with PARP inhibitor and the control plant material to reduce TTC is measured by the procedure described above.

The TTC reducing capacity (as for instance measured by the O.D. at 485 nm) for plant material incubated with PARP inhibitor (TTC-INH) is compared with the TTC reducing capacity of the control plant material incubated without PARP inhibitor (TTC-CON) and a ratio (E) is calculated as follows:

$$E = \text{TTC-INH} / \text{TTC-CON}$$

The value E is a measure of the quality and fitness of the plant material, for example explants to be transformed. It is believed that those tissues, wherein the E value is larger than or equals 1, are healthy tissues, which are particularly suitable as starting material for transformation.

The modified TTC-procedure thus allows to select those types of (cultured) plant material especially appropriate for use in a transformation procedure,

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particularly the procedures of this invention that include the use of a PARP inhibitor.

As the quality of plant material will also be affected by the particular culture conditions used prior to transformation (especially cells, tissues or explants derived from plants recalcitrant to transformation) the assay of this invention is further useful to identify suitable culture conditions to obtain suitable starting plant material. Thus it has been found by the inventor that, when culturing plant material from corn, it is preferred to include proline, preferably at a concentration of about 8mM, simultaneously with the PARP inhibitor, in the culture medium.

As already mentioned, incubation of cells or tissues in the presence of a PARP inhibitor for longer than 1 to 2 days leads to a general reduction in cell metabolism, particularly a reduction in the electron flow in the mitochondrial electron transport chain (after the initial increase, characteristic of healthy cells or tissues, during the first day). The period of time required to reduce the metabolism to an optimal level (for the purpose of improving the qualitative aspect of transformation) is that period after which a decrease in TTC-reducing capacity between 20% and 50%, preferably between 30 % and 40%, particularly about 35%, is achieved for plant material incubated with a PARP inhibitor (e.g. niacinamide) when compared to control plant material incubated without the PARP inhibitor (i.e. the period after which the E value is between 0.5 and 0.8, preferably is between 0.6 and 0.7, particularly is about 0.65).

It is clear that the assays of this invention can be readily adapted by one skilled in the art of the field, for example to suit the needs of the particular cell type, tissue or explant or of the particular species from which the cells, tissues or explants are derived. Furthermore the assay can be adapted to assay a



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peculiar aspect of fitness of cells, tissue, explant or organism. For instance, it is possible to apply a type of stress different from osmotic or salt stress, such as stress brought about by extreme temperatures, by sublethal treatment with chemicals (e.g. herbicides, heavy metals) or by irradiation with UV. Furthermore, other types of PARP inhibitors, as mentioned before may be used, within the indicated concentration ranges. Although it is believed that for the purpose of the assays defined here, TTC is the most suited substrate, other indicator molecules, such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium) can be used to measure the electron flow in the mitochondrial electron transport chain downstream of the "ubiquinone pool".

Unless otherwise indicated all experimental procedures for manipulating recombinant DNA were carried out by the standardized procedures described in Sambrook et al., 1989, "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, and Ausubel et al, 1994, "Current Protocols in Molecular Biology", John Wiley & Sons.

The polymerase chain reactions ("PCR") were used to clone and/or amplify DNA fragments. PCR with overlap extension was used in order to construct chimeric genes (Horton et al, 1989, Gene 77:61-68; Ho et al, 1989, Gene 77:51-59).

All PCR reactions were performed under conventional conditions using the Vent<sup>TM</sup> polymerase (Cat. No. 254L - Biolabs New England, Beverley, MA 01915, U.S.A.) isolated from Thermococcus litoralis (Neuner et al., 1990, Arch.Microbiol. 153:205-207). Oligonucleotides were designed according to known rules as outlined for example by Kramer and Fritz (1968, Methods in Enzymology 154:350), and synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981, Tetrahedron Letters 22:1859) on an applied Biosystems 380A DNA synthesizer (Applied Biosystems B.V., Maarsse,

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Netherlands). In the examples MS medium means Murashige and Skoog medium (Murashige and Skoog, 1962, Physiol. Plant 15:473-479).

In the following examples, reference will be made to the following sequence listing and figures:

#### Sequence Listing

SEQ ID NO 1 : T-DNA of plasmid pTHW107

SEQ ID NO 2: plasmid pTS172

SEQ ID NO 3: PT72 promoter contained in plasmid pTS772

SEQ ID No 4 : plasmid pVE136

SEQ ID No 5 : T-DNA of plasmid pTHW142

#### Examples

##### Example 1 : Tissue culture of wheat embryogenic callus and Brassica napus hypocotyl explants in media containing a PARP inhibitor.

Wheat embryogenic callus was cultured on W2 medium (see Example 2). When niacinamide was added as PARP-inhibitor to the medium at a concentration of 250 mg/l (approx. 2 mM) it was observed that after 4 days the growth of the tissue was slowed down considerably (to approximately 30% of the normal rate after 4 weeks) but the tissue remained viable for extended periods of time (i.e. at least one month). If niacinamide was subsequently removed from the medium the tissue started to grow normally again. It was also observed that after 4-5 days of incubation of the plant tissue with niacinamide, the TTC-reducing capacity (Towill and Mazur, 1975, Can J.Bot. 53:1097-1102) of the tissue was substantially decreased probably indicating a reduction of the production of free radicals and decreased mitochondrial electron transport.

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Similar observations were made when Brassica napus hypocotyl explants were cultured on A5 medium (see Example 3) containing 250 mg/l niacinamide. It was also observed that, in Brassica napus tissue cultured on medium containing niacinamide, no anthocyanin was produced; normally anthocyanin in tissue culture is produced in stress conditions. In addition it was observed that after 4-5 days of incubation of the plant tissue with niacinamide, the concentrations of hydroxyl free radical and dehydroascorbate in the explants were drastically decreased.

It was also observed that, after a 4 day incubation in niacinamide containing medium, the percentage of cultured cells that were in G2 phase of the cell cycle was considerably increased (up to 45 % of all cells in the culture).

The above observations are interpreted as indicating that treating cultured cells with a PARP inhibitor for about 4-5 days generally results in :

- 1) a significant reduction of the response of the cultured cells to stress as measured for instance by free radical and/or anthocyanin production , and
- 2) a reduction of the general metabolism of the cultured cells to a very basic level as indicated by the fact that the tissue growth was slowed down, and the TTC reducing capacity was decreased while the tissue remained viable.

It is inferred that under these conditions many genes in cells (e.g. cultured cells) that would normally be switched on in response to stress (such as during transformation conditions) will in fact no longer be induced. It is expected that in such cells which only display a very basic metabolism, mainly general "housekeeping genes", i.e. genes that are expressed in any cell irrespective of its differentiated state or metabolic or physiological condition, are expressed.

As it is believed that foreign DNA is preferably inserted in portions of the genome that are transcriptionally active it follows that treatment with PARP inhibitors will condition eucaryotic cells to incorporate any foreign DNA

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preferentially in genomic regions which are transcribed in all cells and not in regions of the genome which would only be transcribed under certain conditions, i.e. stress conditions, or during differentiation. This means that the number of locations in which foreign DNA will be integrated, and the concomitant variation in expression profile of the transgene(s), will be reduced. It is further believed that this will enhance integration of foreign genes of interest in such locations which in turn will result in a more reliable and faithful expression of these genes which will be less affected by cell differentiation or cell physiological and biochemical changes due to for instance environmental conditions.

Example 2 : Transformation of wheat with a barnase gene under the control of a stamen-specific promoter using the particle bombardment

The Wheat Spring variety Pavon is grown in a greenhouse or conditioned room at 23-24°C during daytime and 18-20°C at night, with a photoperiod of 16 hours light and 8 hours dark. Developing seeds (white-greenish with white semi-liquid endosperm) were harvested, sterilized by incubation for 1 minute in 70% ethanol followed by 15 minute incubation in 1.3% NaOCl+ 0.1% Tween 20, and washed with sterile water. The sterilized seeds were either used directly or were stored for one day at 4-7°C.

Immature embryos of about 1 mm in size were isolated and were placed, with the scutellum upwards, on callus inducing medium W1 (MS medium supplemented with 3% sucrose, 40 mg/l adenine.SO<sub>4</sub>, 0.5 mg/l thiamine.HCl, 0.5 g/l 2-[N-Morpholino] ethane sulfonic acid (Mes) pH 5.8, 0.5% agarose, 0.5 to 2.5 mg/l CuSO<sub>4</sub>.5H<sub>2</sub>O, 25 mg/l acetylsalicylic acid and 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D)) and were incubated for 3 weeks at 27°C in the dark.

Embryogenic sections of the developing callus were isolated, placed on callus maintenance medium W2 (W1 medium but without acetylsalicylic acid and with

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only 0.5 mg/l  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 1 mg/l 2,4-D), and incubated for 3 weeks at 24-25°C in the light (approx. 20 mEinsteins/s/m<sup>2</sup> (with a photoperiod of 16 hours light and 8 hours dark).

5 About 2 weeks prior to bombardment the calli were cleaned up by removal of non-morphogenic (i.e. the nonembryogenic and nonmeristematic) parts and were subcultured on W2 medium.

10 For bombardment the calli were divided into small pieces with an average maximum diameter of about 2-3 mm. These pieces were placed at the center of a 9 cm Petridish containing W2 medium in a circle with a diameter of approx. 0.5 cm. When required niacinamide (250 mg/l) was added to the W2 medium and the tissue pieces were maintained under these conditions for 4 days after they were bombarded.

15 Bombardment was carried out using the Biolistic PDS-1000/He apparatus (Bio-Rad). Preparation of the microcarriers (0.4-1.2m) and the coating of the microcarriers with DNA was essentially carried out according to the manufacturer's instructions. The Petridishes containing the calli were placed at level 2 of the apparatus and the bombardment was done at 1550 psi.

20 For the transformation experiments the following plasmid DNA was used.

- plasmid pVE136, the sequence of which is given in SEQ ID No 4. This plasmid contains the following chimeric genes:

- P35S-bar-3'nos
- 25 - PCA55-bar-3'nos

in which P35S is the 35S promoter of the Cauliflower Mosaic virus, bar is a DNA encoding phosphinothricin acetyltransferase (EP 242236), 3'nos is the 3' untranslated end of the Agrobacterium T-DNA nopaline synthase gene, PCA55 is a stamen-specific promoter from corn gene CA55 (WO

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9213957), and barnase is a DNA encoding barnase (Hartley, 1988, J.Mol.Biol.202:913-915)

- plasmid pTS172 the sequence of which is given in SEQ ID No 2. This plasmid contains the following chimeric genes:

- 5 - P35S-bar-3'g7
- PE1-barnase-3'nos

in which in which P35S is the 35S promoter of the Cauliflower Mosaic virus, bar is a DNA encoding phosphinothricin acetyltransferase (EP 242236), 3'g7 is the 3' untranslated end of the Agrobacterium T-DNA gene 7, PE1 is a stamen-specific promoter from rice gene E1 (WO 10 9213956), barnase is a DNA encoding barnase (Hartley, 1988, J.Mol.Biol.202:913-915), and 3'nos is the 3' untranslated end of the Agrobacterium T-DNA nopaline synthase gene,

- 15 - plasmid pTS772 which is identical to pTS172 except that the region between nucleotides 2625-4313 of pTS172, containing PE1, is replaced by the sequence of SEQ ID No 3 containing the PT72 promoter. Thus, plasmid pTS772 contains the following chimeric genes:

- P35S-bar-3'g7
- 20 - PT72-barnase-3'nos

in which PT72 is a stamen-specific promoter from rice gene T72 (WO 9213956)

25 The bombarded calli were transferred to selective medium W2 containing 2.5 mg/l phosphinothricin (PPT) and, if necessary, 100 mg/l niacinamide. The calli that were placed on medium containing niacinamide were transferred after 4 days to niacinamide-free W2 medium containing 2.5 mg/l PPT. The cells were cultured at 24-25°C.

After two weeks the calli were subcultivated on W2 medium and after a further two weeks the growing parts of the calli were transferred to regeneration medium W4 (W1 medium but without acetylsalicylic acid and with only 0.5 mg/l

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CuSO<sub>4</sub>.5H<sub>2</sub>O and 0.5 mg/l 2,4-D). Calli were subcultivated every two weeks at which time the nonmorphogenic parts of the calli were removed. When the calli started to form shoots they were transferred to W5 medium (W1 medium with half concentrated MS medium and only 0.5 mg/l CuSO<sub>4</sub>.5H<sub>2</sub>O and without acetylsalicylic acid and 2,4-D, but supplemented with 50 mg/l myo-inositol, 0.25 mg/l pyridoxine.HCl and 0.25 mg/l nicotinic acid) containing 2.5 mg/l PPT. For the rest of the procedure temperature was maintained at a maximum of 24°C. The calli were subcultivated every 3-4 weeks. Once the shoots started to elongate and small roots started to form, the whole calli (or if possible individual shoots) were transferred to 1 liter vessels with W6 medium (half-concentrated MS medium supplemented with 1.5% sucrose, 50 mg/l myo-inositol, 0.25 mg/l pyridoxine.HCl, 0.25 mg/l nicotinic acid, 0.5 mg/l thiamine.HCl, 0.7% agar (Difco) pH 5.8 and 0.5 mg/l CuSO<sub>4</sub>.5H<sub>2</sub>O) containing 2.5 mg/l PPT. Once the shoots and roots had grown out, individual shoots were separated from each other and transferred to 1 l vessels containing W6 medium with 2.5 mg/l PPT. Well developed shoots are tested for PPT resistance by means of the TLC assay (De Block et al, 1987, EMBO 6:2513-2518) or by direct assay of ammonium production in the tissue (see e.g. De Block et al, 1995, Planta 197: 619-626). Transformed shoots were finally transferred to the greenhouse into soil.

For analysis of the results the transformed plants could be subdivided according to the niacinamide treatment of the parent calli during tissue culture. Thus the following groups were distinguished:

<u>Group</u>	<u>Niacinamide treatment</u>
None	No treatment
Before 100	100 mg/l niacinamide for four days prior to bombardment
Before 250	250 mg/l niacinamide for four days prior to bombardment

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Before/After      250 mg/l niacinamide for four days prior to bombardment  
plus 100 mg/l niacinamide for four days after  
bombardment

5      Results of the experiments are presented in Tables 1, 2 and 3. Plants could be  
obtained only from bombarded calli that were treated with niacinamide.  
For the plants that were transformed with plasmid pTS172 it was demonstrated  
that the foreign DNA, comprising the chimeric PE1-barase-3'nos and P35S-  
10      bar-3'g7, was stably incorporated in the wheat genome in 2 to 3 copies on the  
average. The fact that variation in expression profile (e.g. tissue-specificity) of  
the transgenes, especially the chimeric barase genes, was decreased in  
transformed cells was evident from the fact that male-sterile plants that  
otherwise looked completely healthy could be obtained only from bombarded  
calli treated with niacinamide. It is believed that this is due to a more faithful  
15      expression characteristics (i.e. lack of expression) of the integrated stamen-  
selective barase gene in these calli and shoots regenerated from these calli.  
In the control calli undesired expression of the barase gene in tissue cultured  
cells might have prevented recovery of any transformed plants from these calli.  
It is expected that to obtain the same number of male-sterile wheat plants from  
20      control calli a much larger number of calli would have to be bombarded.



**Results of wheat transformation experiments****Table 1 :**

<b>Plasmid pTS172</b>				
<b>Treatment</b>	<b>Nr of bombarded calli</b>	<b>Nr of PPT- resistant calli recovered</b>	<b>Nr of PPT resistant plants recovered</b>	<b>Nr of MS plants recovered</b>
<b>None</b>	<b>60</b>	<b>30</b>	<b>1<sup>a)</sup></b>	<b>0</b>
<b>Before 250</b>	<b>125</b>	<b>30</b>	<b>3</b>	<b>3<sup>b)</sup></b>

a) This plant proved to be fertile and to be transformed only with the chimeric bar gene

b) The obtained plants looked healthy and tillered vigorously

Table 2:

Plasmid pTS772				
Treatment	Nr of bombarded calli	Nr of PPT- resistant calli recovered	Nr of PPT resistant plants recovered	Nr of MS plants recovered
None	250	22	0	0
Before 250	210	75	7	3 <sup>a)b)</sup>
Before/ After	210	45	6	3 <sup>a</sup>

a) The obtained plants looked healthy and tillered vigorously

b) Only six plants could be analyzed for MS phenotype since one of the plants died prematurely.

**Table 3:**

<b>Plasmid pVE136</b>			
<b>Treatment</b>	<b>Nr of bombarded calli</b>	<b>Nr of PPT resistant plants recovered</b>	<b>Nr of MS plants recovered</b>
<b>None</b>	<b>200</b>	<b>1</b>	<b>0</b>
<b>Before 100</b>	<b>800</b>	<b>8<sup>a)</sup></b>	<b>8</b>

a) The obtained plants looked healthy and tillered vigorously

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Example 3: Transformation of oilseed rape with a barnase gene under the control of a stamen-specific promoter using Agrobacterium mediated transformation.

Hypocotyl explants of Brassica napus were obtained, cultured and transformed essentially as described by De Block et al, 1989, Plant Physiol. 914:694-701 except for the following modifications:

- hypocotyl explants were precultured for 3 days on A2 medium (MS, 0.5 g/l Mes (pH 5.7), 1.2% glucose, 0.5% agarose, 1 mg/l 2,4-D, 0.25 mg/l naphthalene acetic acid (NAA), 1 mg/l 6-benzylaminopurine (BAP)), and then transferred to the A2 medium with or without niacinamide for another 4 days.

- infection medium A3 was MS, 0.5 g/l Mes (pH 5.7), 1.2% glucose, 0.1 mg/l NAA, 0.75 mg/l BAP, 0.01 mg/l giberellinic acid (GA3)

- selection medium A5 was 0.5 g/l Mes (pH 5.7), 1.2 % glucose, 40 mg/l adenine.SO<sub>4</sub>, 0.5 g/l polyvinyl-polypyrrolidone (PVP), 0.5% agarose, 0.1 mg/l NAA, 0.75 mg/l BAP, 0.01 mg/l GA3, 250 mg/l carbenicillin, 250 mg/l triacillin, 5 mg/l AgNO<sub>3</sub>.

- regeneration medium A6 was MS, 0.5 g/l Mes (pH 5.7), 2% sucrose, 40 mg/l adenine.SO<sub>4</sub>, 0.5 g/l PVP, 0.5% agarose, 0.0025 mg/l BAP, 250 mg/l triacillin.

- healthy shoots were transferred to 1 liter vessels containing rooting medium which was either A8 or A9; A8 consists of 100-130 ml half concentrated MS, 1% sucrose (pH 5.0), 1 mg/l isobutyric acid (IBA), 100 mg/l triacillin added to 300 ml perlite (final pH 6.2); A9 consists of half concentrated MS, 1.5% sucrose (pH 5.8) solidified with agar (0.6%)

Hypocotyl explants (with or without niacinamide treatment) were infected with Agrobacterium tumefaciens strain C58C1Rif carrying T-DNA vector pTHW107 and a helper Ti-plasmid pMP90 (Koncz and Schell, 1986, Mol.Gen.Genet. 204:383-396)(or a derivative thereof).

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Plasmid pTHW107 is a vector carrying a T-DNA comprising the following chimeric genes :

- PTA29-barnase-3'g7
- PSSU-bar-3'nos

5 in which PTA29 is the promoter of the TA29 gene of tobacco (EP 344029) and PSSU is the promoter of the gene of Arabidopsis thaliana encoding the small subunit of Rubisco. The complete sequence of the T-DNA of pTHW107 is presented in SEQ ID No 1.

10 Where required niacinamide (250 mg/l) was added to the media for the last 4 days prior to infection with Agrobacterium. Plants regenerated from transformed calli obtained on niacinamide cultured cells were observed to have a low copy number as well as to display less variation in the expression profile of the transgenes (results summarized in Table 4). Five plants regenerated  
15 from the calli obtained by transformation including niacinamide and five plants regenerated from the calli obtained by conventional transformation without niacinamide inclusion, were analyzed by Southern hybridization to determine the copy number of the transgenes, and were further analyzed for reproductive phenotype. In the non-treated group, a substantial number of regenerated  
20 plants proved not to have a transgene integrated in their nuclear DNA.

Table 4:

Treatment	Id. No.	Vegetative phenotype <sup>a</sup>	Reproductive phenotype <sup>b</sup>	Copy No. of the transgenes <sup>c</sup>	Phenotype of the F1-progeny <sup>d</sup>
no treatment	1	stressed	sterile	3	stressed/sterile
	2	stressed	sterile	4-6	ND
	3	stressed	sterile	3	stressed/sterile
	4	normal	sterile	1	normal/sterile
	5	stressed	(bud fall)	ND	ND
Before 250	1	normal	sterile	1	normal/sterile
	2	normal	sterile	3	normal/sterile
	3	normal	sterile	1	ND
	4	normal	sterile	3	ND
	5	normal	sterile	2	ND

- a. Vegetatively stressed plants have a small size and flower early, leaves are oblong and dark green.
- b. Reproductive phenotype regards male sterility; in flowers where the buds fell off prematurely this phenotype was not scored, except where some buds resulted in flowers.
- c. Copy number of the transgenes was estimated by comparative Southern. ND: not determined.
- d. F1-progeny was obtained by pollinating the transformed plants with pollen obtained from an untransformed N90-740 line. F1-Progeny resistant to phosphinotricin was scored for vegetative and reproductive phenotype.

Example 4: Agrobacterium-mediated transformation of oilseed rape using niacinamide in the culture medium.

Hypocotyl explants of Brassica napus were obtained as described in Example 3. Four groups of 200 hypocotyl explants each, were either not treated with niacinamide (indicated in table 4 as NONE), treated with 250 mg/l niacinamide for 1 day prior to infection with Agrobacterium (BEFORE), treated for 2 days during the infection with 250 mg/l niacinamide (DURING), or treated for 1 day after the Agrobacterium infection with 250 mg/l niacinamide (AFTER).

All hypocotyl explants were infected with Agrobacterium tumefaciens strain C58C1Rif carrying T-DNA vector pTHW142 and a helper Ti-plasmid pMP90 (Koncz and Shell, 1986 supra)(or a derivative thereof).

Plasmid pTHW142 is a vector carrying a T-DNA comprising the following chimeric genes:

- PSSU-bar-3'g7
- p35S-uidA-3'35S

In which uidA is a DNA encoding b-glucuronidase (Jefferson et al., 1986, Proc. Natl. Acad. Sci. USA 83, 8447-8451) and 3' 35S is the 3' untranslated end of the cauliflower mosaic virus 35S transcript.

The complete sequence of the T-DNA of pTHW142 is presented in SEQ ID No 5.

After the Agrobacterium infection, hypocotyl explants were transferred to selection medium A5, and if appropriate to A5 medium containing 250 mg/l niacinamide. The hypocotyl explants that were placed on medium containing niacinamide were transferred after 1 day to niacinamide-free selection medium A5. After 5 weeks on selective medium the number of transformed calli was scored. b-glucuronidase expression was verified in the obtained calli using established protocols (Jefferson et al., 1986). The results are summarized in

Table 5. Niacinamide treatment either before or after the Agrobacterium infection significantly increase the transformation efficiency.

Table 5:

Treatment	Transformation frequency <sup>a</sup>	Remarks <sup>b</sup>
NONE	16%	small, green calli
BEFORE	32%	large, green calli
DURING	16%	very small, light green calli
AFTER	29%	large, green calli developing shoots

5

a. Determined as the number of transformed calli (PPT-resistant and GUS-positive) developing per 100 hypocotyl explants

b. Size determination was as follows:

very small: callus diameter of approximately 1-2 mm

small: callus diameter of approximately 2-3 mm

large: callus diameter of approximately 5 mm

10

All publications cited in this application are hereby incorporated by reference.



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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: PLANT GENETIC SYSTEMS N.V.
- (B) STREET: Plateaustraat 22
- (C) CITY: Ghent
- (E) COUNTRY: Belgium
- (F) POSTAL CODE (ZIP): 9000
- (G) TELEPHONE: 32 9 235 84 58
- (H) TELEFAX: 32 9 223 19 23
- (I) TELEX: 11.361 Pgsgen

(ii) TITLE OF INVENTION: Genetic Transformation using a PARP inhibitor

(iii) NUMBER OF SEQUENCES: 5

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4946 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: T-DNA of plasmid pTHW107

## (ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (1..25)
- (D) OTHER INFORMATION: /label= RB  
/note= "T-DNA right border"

## (ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (97..330)
- (D) OTHER INFORMATION: /label= 3'g7  
/note= "3' untranslated region containing the

polyadenylation signal of gene 7 of Agrobacterium T-DNA "

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## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (331..882)

(D) OTHER INFORMATION:/label= bar

/note= "region coding for phosphinothricin acetyl  
transferase"

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (883..2608)

(D) OTHER INFORMATION:/label= PSSU

/note= "promoter region of Rubisco small subunit gene of  
Arabidopsis thali..."

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (2658..3031)

(D) OTHER INFORMATION:/label= 3'nos

/note= "3' untranslated region containing the  
polyadenylation signal of the nopaline synthase gene of Agrobacterium  
T-DNA"

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (3032..3367)

(D) OTHER INFORMATION:/label= barnase

/note= "region coding for barnase"

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (3368..4876)

(D) OTHER INFORMATION:/label= PTA29

/note= "promoter region of TA29 gene of Nicotiana tabacum"

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (4922..4946)

(D) OTHER INFORMATION:/label= LB

/note= "T-DNA left border"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

45	AATTACAACG GTATATATCC TGCCAGTACT CGGCCGTCGA ACTCGGCCGT CGAGTACATG	60
	GTCGATAAGA AAAGGCAATT TGTAGATGTT AATTCCCATC TTGAAAGAAA TATAGTTTAA	120
	ATATTTATTG ATAAAATAAC AAGTCAGGTA TTATAGTCCA AGCAAAAACA TAAATTTATT	180
50	GATGCAAGTT TAAATTCAGA AATATTTCAA TAACTGATTA TATCAGCTGG TACATTGCCG	240
	TAGATGAAAG ACTGAGTGCG ATATTATGTG TAATACATAA ATTGATGATA TAGCTAGCTT	300
55	AGCTCATCGG GGGATCCTAG ACGCGTGAGA TCAGATCTCG GTGACGGGCA GGACCGGACG	360

	GGGCGGTACC GGCAGGCTGA AGTCCAGCTG CCAGAAACCC ACGTCATGCC AGTTCCCGTG	420
5	CTTGAAGCCG GCCGCCCGCA GCATGCCGCG GGGGGCATAT CCGAGCGCCT CGTGCATGCG	480
	CACGCTCGGG TCGTTGGGCA GCCCGATGAC AGCGACCACG CTCTTGAAGC CCTGTGCCTC	540
	CAGGGACTTC AGCAGGTGGG TG TAGAGCGT GGAGCCAGT CCCGTCCGCT GGTGGCGGGG	600
10	GGAGACGTAC ACGGTCGACT CGGCCGTCCA GTCGTAGGCG TTGCGTGCCT TCCAGGGGCC	660
	CGCGTAGGCG ATGCCGGCGA CCTCGCCGTC CACCTCGGCG ACGAGCCAGG GATAGCGCTC	720
15	CCGCAGACGG ACGAGGTCGT CCGTCCACTC CTGCGGTTCC TCGGCTCGG TACGGAAGTT	780
	GACCGTGCTT GTCTCGATGT AGTGGTTGAC GATGGTGCAG ACCGCCGGCA TGTCCGCCCTC	840
	GGTGGCACGG CGGATGTCGG CCGGGCGTCG TTCTGGGTCC ATTGTTCTTC TTTACTCTTT	900
20	GTGTGACTGA GGTTTGGTCT AGTGCTTTGG TCATCTATAT ATAATGATAA CAACAATGAG	960
	AACAAGCTTT GGAGTGATCG GAGGGTCTAG GATACATGAG ATTCAAGTGG ACTAGGATCT	1020
25	ACACCGTTGG ATTTTGAGTG TGGATATGTG TGAGGTTAAT TTTACTTGGT AACGGCCACA	1080
	AAGGCCAAG GAGAGGTGTT GAGACCCTTA TCGGCTTGAA CCGCTGGAAT AATGCCACGT	1140
	GGAAGATAAT TCCATGAATC TTATCGTTAT CTATGAGTGA AATTGTGTGA TGGTGGAGTG	1200
30	GTGCTTGCTC ATTTTACTTG CCTGGTGGAC TTGGCCCTTT CTTTATGGGG AATTTATATT	1260
	TTACTTACTA TAGAGCTTTC ATACCTTTTT TTTACCTTGG ATTTAGTTAA TATATAATGG	1320
35	TATGATTCAT GAATAAAAT GGGAAATTTT TGAATTTGTA CTGCTAAATG CATAAGATTA	1380
	GGTGAAACTG TGGAATATAT ATTTTTTCA TTTAAAAGCA AAATTTGCCT TTTACTAGAA	1440
	TTATAAATAT AGAAAAATAT ATAACATTCA AATAAAAATG AAAATAAGAA CTTTCAAAAA	1500
40	ACAGAACTAT GTTTAATGTG TAAAGATTAG TCGCACATCA AGTCATCTGT TACAATATGT	1560
	TACAACAAGT CATAAGCCCA ACAAAGTTAG CACGTCTAAA TAACTAAAG AGTCCACGAA	1620
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	GCTAAACAAA GTCCAAAAA AACTTCTCAA GTCTCCATCT TCCTTTATGA ACATTGAAAA	1740
	CTATACACAA AACAAATCAG ATAAATCTCT TTCTGGGCCT GTCTTCCCAA CCTCCTACAT	1800
50	CACTTCCCTA TCGGATTGAA TGTTTACTT GTACCTTTTC CGTTGCAATG ATATTGATAG	1860
	TATGTTTGTG AAAACTAATA GGGTTAACAA TCGAAGTCAT GGAATATGGA TTTGGTCCAA	1920
55	GATTTTCCGA GAGCTTTCTA GTAGAAAGCC CATCACCAGA AATTTACTAG TAAATAAAT	1980

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	CACCAATTAG	GTTTCTTATT	ATGTGCCAAA	TTCAATATAA	TTATAGAGGA	TATTTCAAAT	2040
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5	GATATTCAAC	TTTAAAAATT	CGATCAGTGT	GGAATTGTAC	AAAAATTTGG	GATCTACTAT	2160
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	ATATTTGTTT	TGGCCATGCA	CCAATCATT	GTTTAGTGTA	ATACTTTGAT	TTTGTCAAAT	2280
10	ATAATGTGTT	GTGTATATTT	GTATAAGAA	TTCTTTGACC	ATATACACAC	ACACATATAT	2340
	ATATATATAT	ATATATTATA	TATCATGCAC	TTTTAATTGA	AAAAATAATA	TATATATATA	2400
15	TAGTGCAATTT	TTTCTAACAA	CCATATATGT	TGCGATTGAT	CTGCAAAAAT	ACTGCTAGAG	2460
	TAATGAAAAA	TATAATCTAT	TGCTGAAATT	ATCTCAGATG	TTAAGATTTT	CTTAAAGTAA	2520
	ATTCTTTCAA	ATTTTAGCTA	AAAGTCTTGT	AATAACTAAA	GAATAATACA	CAATCTCGAC	2580
20	CACGGAAAAA	AAACACATAA	TAAATTTGAA	TTTCGACCGC	GGTACCCGGA	ATTCGAGCTC	2640
	GGTACCCGGG	GATCTTCCCG	ATCTAGTAAC	ATAGATGACA	CCGCGCGCGA	TAATTTATCC	2700
25	TAGTTTGCGC	GCTATATTTT	GTTTTCTATC	GCGTATTAAA	TGTATAATTG	CGGGACTCTA	2760
	ATCATAAAAA	CCCATCTCAT	AAATAACGTC	ATGCATTACA	TGTTAATTAT	TACATGCTTA	2820
	ACGTAATTCA	ACAGAAATTA	TATGATAATC	ATCGCAAGAC	CGGCAACAGG	ATTCAATCTT	2880
30	AAGAACTTTT	ATTGCCAAT	GTTTGAACGA	TCTGCTTCGG	ATCCTCTAGA	GCCGGAAAGT	2940
	GAAATTGACC	GATCAGAGTT	TGAAGAAAAA	TTTATTACAC	ACTTTATGTA	AAGCTGAAAA	3000
35	AAACGGCCTC	CGCAGGAAGC	CGTTTTTTTC	GTTATCTGAT	TTTTGTAAAG	GTCTGATAAT	3060
	GGTCCGTTGT	TTTGTAATC	AGCCAGTCGC	TTGAGTAAAG	AATCCGGTCT	GAATTTCTGA	3120
	AGCCTGATGT	ATAGTTAATA	TCCGCTTCAC	GCCATGTTTC	TCCGCTTTTG	CCCGGGAGTT	3180
40	TGCCTTCCCT	GTTTGAGAAG	ATGTCTCCGC	CGATGCTTTT	CCCCGGAGCG	ACGTCTGCAA	3240
	GGTTCCCTTT	TGATGCCACC	CAGCCGAGGG	CTTGTGCTTC	TGATTTTGTA	ATGTAATTAT	3300
45	CAGGTAGCTT	ATGATATGTC	TGAAGATAAT	CCGCAACCCC	GTCAAACGTG	TTGATAACCG	3360
	GTACCATGGT	AGCTAATTTT	TTTAAGTAAA	AACCTTTGATT	TGAGTGATGA	TGTTGTACTG	3420
	TTACACTTGC	ACCACAAGGG	CATATATAGA	GCACAAGACA	TACACAACAA	CTTGCAAAAC	3480
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	TTAAGGTTTC	ATGTATTAAT	TTGTTGCAAA	CATGGACTTA	GTGTGAGGAA	AAAGTACCAA	3600
55	AATTTTGTCT	CACCCTGATT	TCAGTTATGG	AAATTACATT	ATGAAGCTGT	GCTAGAGAAG	3660

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5  
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ATGTTTATTC TAGTCCAGCC ACCCACCTTA TGCAAGTCTG CTTTGTAGCTT GATTCAAAAA 3720  
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AGGATGTATA TATTAGTACA TAAAAAATCA TGTTTGAATC ATCTTTCATA AAGTGACAAG 3840  
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CAGATTGTGA CATGGAAAAC AAAAAGTCCT CTGATAGAAG TCGCAAAGTA TCACAATTTT 4140  
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CCTAAAAACA GCATATGGTA GTTTCTAGGG AATCTAAATC ACTAAAATTA ATAAAAGAAG 4620  
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AATGTATATT ATATGCATAA TTTATATATT AAATGTGTAT AATCATGTAT AATCAATGTA 4800  
TAATCTATGT ATATGGTTAG AAAAAGTAAA CAATTAATAT AGCCGGCTAT TTGTGTAAAA 4860  
ATCCCTAATA TAATCGCGAC GGATCCCCGG GAATTCCGGG GAAGCTTAGA TCCATGGAGC 4920  
CATTTACAAT TGAATATATC CTGCCG 4946

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6548 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: plasmid pTS172

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (2019..2288)

(D) OTHER INFORMATION:/label= 3'nos

/note= "3' untranslated region containing the  
polyadenylation signal of the nopaline synthase gene of Agrobacterium  
T-DNA"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (2289..2624)

(D) OTHER INFORMATION:/label= barnase

/note= "region coding for barnase"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:complement (2625..4313)

(D) OTHER INFORMATION:/label= PE1

/note= "promoter region of E1 gene of rice"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:4336..5710

(D) OTHER INFORMATION:/label= P35S

/note= "35S promoter region of Cauliflower mosaic virus"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:5711..6262

(D) OTHER INFORMATION:/label= bar

/note= "region coding for phosphinothricin acetyl  
transferase"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:6263..6496

(D) OTHER INFORMATION:/label= 3'g7

/note= "3' untranslated region containing the  
polyadenylation signal of gene 7 of Agrobacterium T-DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTCAAGCT TGACGTCAGG TGGCACTTTT CGGGGAAATG TGCGCGGAAC CCCTATTTGT 60

TTATTTTCT AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC CTGATAAATG 120

CTTCAATAAT ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT CGCCCTTATT 180

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	CCCTTTTTTG	CGGCATTTTG	CCTTCCTGTT	TTTGCTCACC	CAGAAACGCT	GGTGAAAGTA	240
5	AAAGATGCTG	AAGATCAGTT	GGGTGCACGA	GTGGGTAC	TCGAACTGGA	TCTCAACAGC	300
	GGTAAGATCC	TTGAGAGTTT	TCGCCCCGAA	GAACGTTTTC	CAATGATGAG	CACTTTTAAA	360
	GTTCTGCTAT	GTGGCGCGGT	ATTATCCCGT	ATTGACGCCG	GGCAAGAGCA	ACTCGGTGCG	420
10	CGCATACT	ATTCTCAGAA	TGACTTGTT	GAGTACTCAC	CAGTCACAGA	AAAGCATCTT	480
	ACGGATGGCA	TGACAGTAAG	AGAATTATGC	AGTGCTGCCA	TAACCATGAG	TGATAACACT	540
15	GCGGCCAACT	TACTTCTGAC	AACGATCGGA	GGACCGAAGG	AGCTAACCGC	TTTTTTGCAC	600
	AACATGGGGG	ATCATGTAAC	TCGCCTTGAT	CGTTGGGAAC	CGGAGCTGAA	TGAAGCCATA	660
	CCAAACGACG	AGCGTGACAC	CACGATGCCT	GTAGCAATGG	CAACAACGTT	GCGCAAATA	720
20	TTAACTGGCG	AACTACTTAC	TCTAGCTTCC	CGGCAACAAT	TAATAGACTG	GATGGAGGCG	780
	GATAAAGTTG	CAGGACCACT	TCTGCGCTCG	GCCCTTCCGG	CTGGCTGGTT	TATTGCTGAT	840
25	AAATCTGGAG	CCGGTGAGCG	TGGGTCTCGC	GGTATCATTG	CAGCACTGGG	GCCAGATGGT	900
	AAGCCCTCCC	GTATCGTAGT	TATCTACACG	ACGGGGAGTC	AGGCAACTAT	GGATGAACGA	960
	AATAGACAGA	TCGCTGAGAT	AGGTGCCTCA	CTGATTAAGC	ATTGGTAACT	GTCAGACCAA	1020
30	GTTTACTCAT	ATATACTTTA	GATTGATTTA	AACTTCATT	TTTAATTTAA	AAGGATCTAG	1080
	GTGAAGATCC	TTTTTGCTC	GAGTCTCATG	ACCAAAATCC	CTTAACGTGA	GTTTTCTGTC	1140
35	CACTGAGCGT	CAGACCCCGT	AGAAAAGATC	AAAGGATCTT	CTTGAGATCC	TTTTTTCTG	1200
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40	AATACTGTCC	TTCTAGTGTA	GCCGTAGTTA	GGCCACCACT	TCAAGAACTC	TGTAGACCCG	1380
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45	TGTCTTACCG	GGTTGGACTC	AAGACGATAG	TTACCGGATA	AGGCGCAGCG	GTCGGGCTGA	1500
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55	TGCTCGTCAG	GGGGGCGGAG	CCTATGGAAA	AACGCCAGCA	ACGCGGCCTT	TTTACGGTTC	1800

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	CTGGCCTTTT	GCTGGCCTTT	TGCTCACATG	TTCTTTCTCTG	CGTTATCCCC	TGATTCTGTG	1860
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5	CGCAGCGAGT	CAGTGAGCGA	GGAAGCGGAA	GAGCGCCCAA	TACGCAAACC	GCCTCTCCCC	1980
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10	CCGCGCGCGA	TAATTTATCC	TAGTTTGCGC	GCTATATTTT	GTTTTCTATC	GCGTATTAAA	2100
	TGTATAATTG	CGGGACTCTA	ATCATAAAAA	CCCATCTCAT	AAATAACGTC	ATGCATTACA	2160
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15	CGGCAACAGG	ATTCAATCTT	AAGAACTTTT	ATTGCCAAAT	GTTTGAACGA	TCTGCTTCGG	2280
	AGGTTACCTT	ATCTGATTTT	TGTAAAGGTC	TGATAATGGT	CCGTTGTTTT	GTAATCAGC	2340
20	CAGTCGCTTG	AGTAAAGAAT	CCGGTCTGAA	TTTCTGAAGC	CTGATGTATA	GTTAATATCC	2400
	GCTTCACGCC	ATGTTCTGTC	GCTTTTGCCC	GGGAGTTTGC	CTTCCCTGTT	TGAGAAGATG	2460
	TCTCCGCCGA	TGCTTTTCCC	CGGAGCGACG	TCTGCAAGGT	TCCCTTTTGA	TGCCACCCAG	2520
25	CCGAGGGCTT	GTGCTTCTGA	TTTTGTAATG	TAATTATCAG	GTAGCTTATG	ATATGTCTGA	2580
	AGATAATCCG	CAACCCCGTC	AAACGTGTTG	ATAACCGGTA	CCATCGCGAC	GGCTTGATGG	2640
30	ATCTCTTGCT	GGACACCGGG	ATGCTAGGAT	GGGTTATCGT	GGCCGGCGTG	CGTGTGTGGC	2700
	TTTTGTAGGC	GCCGGCGACG	GCGGGGGCAA	TGTGGCAGGT	GAGTCACGGT	GCAAGCGTGC	2760
	GCAAGTGACT	GCAACAACCA	AGGACGGTCA	TGGCGAAAGC	ACCTCACGCG	TCCACCGTCT	2820
35	ACAGGATGTA	GCAGTAGCAC	GGTGAAAGAA	GTGTTGTCCC	GTCCATTAGG	TGCATTCTCA	2880
	CCGTTGGCCA	GAACAGGACC	GTTCAACAGT	TAGGTTGAGT	GTAGGACTTT	TACGTGGTTA	2940
40	ATGTATGGCA	AATAGTAGTA	AATTTTGCCC	CCATTGGTCT	GGCTGAGATA	GAACATATTC	3000
	TGGAAAGCCT	CTAGCATATC	TTTTTTGACA	GCTAAACTTT	GCTTCTTGCC	TTCTTGGTCT	3060
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45	CCTTCAACGG	CTCAATCCCC	ACAGGCCAAG	CTATCCTTTC	CTTGGCAGTA	TAGGCTCCTT	3180
	GAGAGATTAT	ACTACCATTT	TTAAGTGCTT	ATAAAGACGA	TGCTCTCTAA	CCAGATCGAT	3240
50	CAGAAACACA	AAGTTTTAGC	AGCGTAATAT	CCCACACACA	TACACACACG	AAGCTATGCC	3300
	TCCTCATTTT	CCGAGAGATT	CTGACAGTGA	CCAGAATGTC	AGAATGCCAT	TTCATGGGCA	3360
	CAAGTCGATC	CACAAGCTTC	TTGGTGGAGG	TCAAGGTGTG	CTATTATTAT	TCGCTTTCTA	3420
55	GGAAATTATT	CAGAATTAGT	GCCTTTTATC	ATAACTTCTC	TCTGAGCCGA	TGTGGTTTTG	3480



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	GATTTTCATTG TTGGGAGCTA TGCAAGTTGCG GATATTCTGC TGTGGAAGAA CAGGAACTTA	3540
5	TCTGCGGGGG TCCTTGCTGG GGCAACATTG ATATGGTTCC TGTTGATGT AGTAGAATAC	3600
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	TCAAATGCCG CACCACTCTT GGACAGGTAT TAGCTTTATT TCCTGTGGAG ATGGTAGAAA	3720
10	ACTCAGCTTA CAGAAATGGC ATTTACGTA GTATAACGCA AGACATTAGG TACTAAAACT	3780
	CAACTAACTG TTTCCGAATT TCAGGGCCCC TCCAAGGATC CCAGAAATCA TCATCTCTGA	3840
15	ACATGCCTTC AGAGAAATGG CATTGACCGT CCATTACAAA CTAACGTACA CTGTATCTGT	3900
	TCTTTACGAC ATTGCATGTG GAAAGGATCT GAAGAGATT CTCTGGTAC ATAATAATCT	3960
	ACTCCTTTGC TACGTTAATA AGAGATGTAA AAACATGCAA CAGTCCAGT GCCAACATTG	4020
20	TCCAAGGATT GTGCAATTCT TTCTGGAGCG CTAAAATTGA CCAGATTAGA CGCATCAGAA	4080
	TATTGAATTG CAGAGTTAGC CAATAATCCT CATAATGTTA ATGTGCTATT GTTGTTCCT	4140
25	ACTCAATATA GTTCTGGACT AACAATCAGA TTGTTTATGA TATTAAGGTG GTTGGATCTC	4200
	TATTGGTATT GTCGGCGATT GGAAGTTCTT GCAGCTTGAC AAGTCTACTA TATATTGGTA	4260
	GGTATTCCAG ATAAATATTA AATTTTAATA AAACAATCAC ACAGAAGGAT CTGCGGCCGC	4320
30	TAGCCTAGGC CCGGGCCAC AAAAATCTGA GCTTAACAGC ACAGTTGCTC CTCTCAGAGC	4380
	AGAATCGGGT ATTCAACACC CTCATATCAA CTACTACGTT GTGTATAACG GTCCACATGC	4440
35	CGGTATATAC GATGACTGGG GTTGTACAAA GGCGGCAACA AACGGCGTTC CCGGAGTTGC	4500
	ACACAAGAAA TTTGCCACTA TTACAGAGGC AAGAGCAGCA GCTGACGCGT ACACAACAAG	4560
	TCAGCAAACA GACAGGTTGA ACTTCATCCC CAAAGGAGAA GCTCAACTCA AGCCCAAGAG	4620
40	CTTTGCTAAG-GCCCTAACAA GCCCACAAA GCAAAAAGCC CACTGGCTCA CGCTAGGAAC	4680
	CAAAAGGCCC AGCAGTGATC CAGCCCCAAA AGAGATCTCC TTTGCCCCGG AGATTACAAT	4740
45	GGACGATTC CTCTATCTTT ACGATCTAGG AAGGAAGTTC GAAGGTGAAG GTGACGACAC	4800
	TATGTTCAAC ACTGATAATG AGAAGGTTAG CCTCTTCAAT TTCAGAAAGA ATGCTGACCC	4860
	ACAGATGGTT AGAGAGGCCT ACGCAGCAGG TCTCATCAAG ACGATCTACC CGAGTAACAA	4920
50	TCTCCAGGAG ATCAAATACC TTCCAAGAA GGTAAAGAT GCAGTCAAAA GATTCAGGAC	4980
	TAATTGCATC AAGAACACAG AGAAAGACAT ATTTCTCAAG ATCAGAAGTA CTATTCCAGT	5040
55	ATGGACGATT CAAGGCTTGC TTCATAAACC AAGGCAAGTA ATAGAGATTG GAGTCTCTAA	5100

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AAAGGTAGTT CCTACTGAAT CTAAGGCCAT GCATGGAGTC TAAGATTCAA ATCGAGGATC 5160  
TAACAGAACT CGCCGTGAAG ACTGGCGAAC AGTTCATACA GAGTCTTTTA CGACTCAATG 5220  
5 ACAAGAAGAA AATCTTCGTC AACATGGTGG AGCACGACAC TCTGGTCTAC TCCAAAAATG 5280  
TCAAAGATAC AGTCTCAGAA GACCAAAGGG CTATTGAGAC TTTTCAACAA AGGATAATTT 5340  
CGGGAAACCT CCTCGGATTC CATTGCCAG CTATCTGTCA CTTTCATCGAA AGGACAGTAG 5400  
10 AAAAGGAAGG TGGCTCCTAC AAATGCCATC ATTGCGATAA AGGAAAGGCT ATCATTCAAG 5460  
ATGCCTCTGC CGACAGTGGT CCCAAAGATG GACCCCCACC CACGAGGAGC ATCGTGGAAA 5520  
15 AAGAAGACGT TCCAACCACG TCTTCAAAGC AAGTGGATTG ATGTGACATC TCCACTGACG 5580  
TAAGGGATGA CGCACAATCC CACTATCCTT CGCAAGACCC TTCCTCTATA TAAGGAAGTT 5640  
CATTTCATTT GGAGAGGACA CGCTGAAATC ACCAGTCTCT CTCTATAAAT CTATCTCTCT 5700  
20 CTCTATAACC ATGGACCCAG AACGACGCC GGCCGACATC CGCCGTGCCA CCGAGGCGGA 5760  
CATGCCGGCG GTCTGCACCA TCGTCAACCA CTACATCGAG ACAAGCACGG TCAACTCCG 5820  
25 TACCGAGCCG CAGGAACCGC AGGAGTGGAC GGACGACCTC GTCCGTCTGC GGGAGCGCTA 5880  
TCCCTGGCTC GTCGCCGAGG TGGACGGCGA GGTGCCCGGC ATCGCCTACG CGGGCCCTG 5940  
GAAGGCACGC AACGCCTACG ACTGGACGGC CGAGTCGACC GTGTACGTCT CCCCCGCCA 6000  
30 CCAGCGGACG GGAAGTGGCT CCACGCTCTA CACCCACCTG CTGAAGTCCC TGGAGGCACA 6060  
GGGCTTCAAG AGCGTGGTCG CTGTTCATCG GCTGCCCAAC GACCCGAGCG TCGCATGCA 6120  
35 CGAGGCGCTC GGATATGCCC CCCGCGGCAT GCTGCGGGCG GCCGGCTTCA AGCACGGGAA 6180  
CTGGCATGAC GTGGGTTTCT GGCAGCTGGA CTTAGCCTG CCGGTACCGC CCGTCCGGT 6240  
CCTGCCCGTC ACCGAGATCT GAGATCACGC GTTCTAGGAT CCCCCGATGA GCTAAGCTAG 6300  
40 CTATATCATC AATTTATGTA TTACACATAA TATCGCACTC AGTCTTTCAT CTACGGCAAT 6360  
GTACCAGCTG ATATAATCAG TTATTGAAAT ATTTCTGAAT TTAACTTGC ATCAATAAAT 6420  
45 TTATGTTTTT GCTTGGACTA TAATACCTGA CTTGTTATTT TATCAATAAA TATTTAACT 6480  
ATATTTCTTT CAAGATGGGA ATTAACATCT ACAAATTGCC TTTTCTTATC GACCATGTAC 6540  
GTATCGCG 6548

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1601 base pairs

(B) TYPE: nucleic acid

55

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(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: T72 promoter region

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: complement (1..1601)

(D) OTHER INFORMATION: /label= PT72

/note= "promoter region of T72 gene of rice"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGCCGTGAGT	GTCTTCTGCC	GCCGAGGGGC	TCTCGCTCGT	CGTCGATGCC	TGCACGGTGC	60
GTGCGTGTGT	GTCGTGGTGG	TGGTGGCGAT	ACGCGACGCG	AGCTCGATTT	ATAGGAGGGG	120
ATCGAAGGAG	GGGAGCGCGC	GCGGCGAGGC	CCGCGTTGCT	CACCTACGCC	GCGCGCATGC	180
GGCGGACGCG	CGGTCGGCGC	CCGCGCCGGC	CGGGAGGACG	AGGGCGCAAG	CGTGTGAGCC	240
ACCGAACGCG	CGCGCGCGCC	GCGGCGCGAA	CTCTCCATCG	CGTCGCGGCG	AGCCGAGAGC	300
CGACGAGAGC	GTTTCGCGCG	CGCGTTGGG	CCGCGCACAA	GATGGGCCGT	AGCCCTGGGC	360
CTCGTGCCAT	CTTTTTTTTT	CTTTTTTGCC	TTTTTTGGCC	TGGCAATTC	TTTTTGTTTT	420
TAGTCTTTTT	GTGGTGATAA	TGTGTCGTCT	TCCGGTGAAC	TAATTACTC	GTTGATCTTT	480
TTGTGTCCCT	TCGAATATTC	GCAGTGGTAG	AAGATGACTA	CTACTACCAG	TAGTTGATCT	540
CGAATGGCAA	ETTTTGTGCA	GAACTTATTC	CACGGCTATG	TCAGCTTCCA	CTGTGACTAA	600
AAAAACTACG	GCCATCTTTT	GGACTTGTTT	TATCTTGGAA	CTGAACAAAA	AGGACGATCC	660
TGATGTACAC	ACGGCATAGT	TTCCAGCACT	GGATGCCAAG	TTGCCAACTG	TTACCACGAT	720
AATGGAACGA	CGAGATGAGA	TATTATACAA	GTCCAATGGA	TCAAGATCCT	GTGCAGTTGT	780
TATTGTAAC	GTAACCTAAG	CCGTTAACAT	GTACATCACA	TTTCCTACTC	TATCAATGTC	840
TTGTGCGGGT	TGTTTCAAAA	AAACATGTAC	ATCACATGAT	CTAGAACGGA	AGGCCAGGAT	900
ATGAAGTGGT	ACTGCAGCAA	AAACACTGTA	GCAGAGATGT	ACTATTATGC	ATGTACTGTA	960
GCAGTCATCT	AGAGCCGTTG	GATCTGAAAA	CGAATGGACA	TGATTGTGTG	CAGTTGCTAT	1020

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TGTGCAGTTA CAATAGCAAC TGCATTTGAT CTTAATCCAA GTCCAATACA TGCAGAACAG 1080  
TAGCTACGAG CTGGAAAGGA TGCAAATCTG GGTGACACTG ACAGCAACCG TGGAAGAACA 1140  
5 ACAGCAGCAA AGTCCCAGAG GGATGGCAAT TTGAAGGAAT TTAAATACTC TAATATTACT 1200  
CCACCCGTTA AAAAAACAA CTTGCTACGC ATAATATATG TTCGGATTTA TAGCGAGAAG 1260  
10 TTAATTTTTC ATGAGAAGAA GAATATATAT GTAATATGTA CTAGGAGAGT ACTCGCTTCA 1320  
TAAATATAAA TATTCATAAG TTGTCCAGTG AAGATAGCTT TAGAAAAAC TAGTTATTTT 1380  
ATTTGTCAAA TTTTAAATTT TGAAGTAGTT AGATTATCTT TCTAGTAGTT CTGATTGGTT 1440  
15 GAAAATGTTT AGATTTTCAT GTGTTAAGAG TTCCGTATCC TAAAAATAGT AATATAATTT 1500  
TAAATCATAT ATATATATAT ATATATATAT ATATATATAT ATATATATAT ATATATATAT 1560  
TGTTGAACGG TTTGTGCTCT GGTGCTATC CTGTTCTGTG G 1601  
20

## (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:  
25 (A) LENGTH: 6291 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: circular  
  
(ii) MOLECULE TYPE: DNA (genomic)  
30  
(iii) HYPOTHETICAL: NO  
  
(iv) ANTI-SENSE: NO  
  
35 (vi) ORIGINAL SOURCE:  
(A) ORGANISM: plasmid pVE136  
  
(ix) FEATURE:  
40 (A) NAME/KEY: -  
(B) LOCATION: complement (425..687)  
(D) OTHER INFORMATION: /label= 3'nos  
/note= "3'untranslated region containing the  
polyadenylation signal of the nopaline synthase gene of Agrobacterium  
45 T-DNA"  
  
(ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (803..1138)  
(D) OTHER INFORMATION: /label= barnase  
50 /note= "region coding for barnase"  
  
(ix) FEATURE:  
(A) NAME/KEY: -  
(B) LOCATION: complement (1138..2317)  
55 (D) OTHER INFORMATION: /label= PCa55

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/note= "stamen-specific promoter from corn gene CA55"

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:2355..3187

(D) OTHER INFORMATION:/label= P35S

/note= "35S promoter region of Cauliflower mosaic virus"

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:3188..3739

(D) OTHER INFORMATION:/label= bar

/note= "region coding for phosphotransferase"

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:3757..4017

(D) OTHER INFORMATION:/label= 3'nos

/note= "3' untranslated region containing the  
polyadenylation signal of the nopaline synthase gene of Agrobacterium  
T-DNA"

## (ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:699..702

(D) OTHER INFORMATION:/note= "region with unknown  
sequence (may contain up to 15 nucleotides)"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

35	TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA	60
	CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG	120
	TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC	180
40	ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC	240
	ATTGCGCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT	300
	TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT	360
45	TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGAATT CGAGCTCGGT ACCCGGGGAT	420
	CTTCCCAGTC TAGTAACATA GATGACACCG CGCGCGATAA TTTATCCTAG TTTGCGCGCT	480
50	ATATTTTGTT TTCTATCGCG TATTAAATGT ATAATTGCGG GACTCTAATC ATAAAAACCC	540
	ATCTCATAAA TAACGTCATG CATTACATGT TAATTATTAC ATGCTTAACG TAATTCAACA	600
55	GAAATTATAT GATAATCATC GCAAGACCGG CAACAGGATT CAATCTTAAG AAACCTTTATT	660

	GCCAAATGTT TGAACGATCT GCTTCGGATC CTCTAGAGNN NNCCGGAAAG TGAAATTGAC	720
	CGATCAGAGT TTGAAGAAAA ATTTATTACA CACTTTATGT AAAGCTGAAA AAAACGGCCT	780
5	CCGCAGGAAG CCGTTTTTTT CGTTATCTGA TTTTGTAAA GGTCTGATAA TGGTCCGTTG	840
	TTTTGTAAAT CAGCCAGTCG CTTGAGTAAA GAATCCGGTC TGAATTTCTG AAGCCTGATG	900
10	TATAGTTAAT ATCCGCTTCA CGCCATGTTT GTCCGCTTTT GCCCCGGAGT TTGCCTTCCC	960
	TGTTTGAGAA GATGTCTCCG CCGATGCTTT TCCCCGGAGC GACGTCTGCA AGGTTCCTTT	1020
	TTGATGCCAC CCAGCCGAGG GCTTGTGCTT CTGATTTTGT AATGTAATTA TCAGGTAGCT	1080
15	TATGATATGT CTGAAGATAA TCCGCAACCC CGTCAAACGT GTTGATAACC GGTACCATGG	1140
	CTGCAGCTAG TTAGCTCGAT GTATCTTCTG TATATGCAGT GCAGCTTCTG CGTTTTGGCT	1200
20	GCTTTGAGCT GTGAAATCTC GCTTTCAGT CCCTGCGTGT TTTATAGTGC TGTACGTTCTG	1260
	TGATCGTGAG CAAACAGGGC GTGCCTCAAC TACTGGTTTG GTTGGGTGAC AGGCGCCAAC	1320
	TACGTGCTCG TAACCGATCG AGTGAGCGTA ATGCAACATT TTTCTTCTT CTCTCGCATT	1380
25	GGTTTCATCC AGCCAGGAGA CCCGAATCGA ATTGAAATCA CAAATCTGAG GTACAGTATT	1440
	TTTACAGTAC CGTTCGTTCTG AAGGTCTTCG ACAGGTCAAG GTAACAAAAT CAGTTTTTAA	1500
30	TTGTTGTTTC AGATCAAAGA AAATTGAGAT GATCTGAAGG ACTTGGACCT TCGTCCAATG	1560
	AAACACTTGG ACTAATTAGA GGTGAATTGA AAGCAAGCAG ATGCAACCGA AGGTGGTGAA	1620
	AGTGGAGTTT CAGCATTGAC GACGAAAACC TTCGAACGGT ATAAAAAGA AGCCGCAATT	1680
35	AAACGAAGAT TTGCCAAAA GATGCATCAA CCAAGGGAAG ACGTGCATAC ATGTTTGATG	1740
	AAAACCTCGTA AAAACTGAAG TACGATTCCC CATTCCCCTC CTTTTCTCGT TTCTTTTAAC	1800
40	TGAAGCAAAG AATTTGTATG TATTCCCTCC ATTCCATATT CTAGGAGGTT TTGGCTTTTC	1860
	ATACCCTCCT CCATTTCAAA TTATTTGTCA TACATTGAAG ATATACACCA TTCTAATTTA	1920
	TACTAAATTA CAGCTTTTAG ATACATATAT TTTATTATAC ACTTAGATAC GTATTATATA	1980
45	AAACACCTAA TTTAAAATAA AAAATTATAT AAAAAAGTGA TCTAAAAAAT CAAAATACGA	2040
	CATAATTTGA AACGGAGGGG TACTACTTAT GCAAACCAAT CGTGGTAACC CTAAACCCTA	2100
50	TATGAATGAG GCCATGATTG TAATGCACCG TCTGATTAC CAAGATATCA ATGGTCAAAG	2160
	ATATACATGA TACATCCAAG TCACAGCGAA GGCAAATGTG ACAACAGTTT TTTTACCAG	2220
	AGGGACAAGG GAGAATATCT ATTCAGATGT CAAGTTCCCG TATCACACTG CCAGGTCCTT	2280
55	ACTCCAGACC ATCTTCCGGC TCTATTGATG CATACCAGGA ATTGATCTAG AGTCGACCTG	2340

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	CAGGCATGCA	AGCTCCTACG	CAGCAGGTCT	CATCAAGACG	ATCTACCCGA	GTAACAATCT	2400
	CCAGGAGATC	AAATACCTTC	CCAAGAAGGT	TAAAGATGCA	GTCAAAAGAT	TCAGGACTAA	2460
5	TTGCATCAAG	AACACAGAGA	AAGACATATT	TCTCAAGATC	AGAAGTACTA	TTCCAGTATG	2520
	GACGATTCAA	GGCTTGCTTC	ATAAACCAAG	GCAAGTAATA	GAGATTGGAG	TCTCTAAAAA	2580
10	GGTAGTTCCT	ACTGAATCTA	AGGCCATGCA	TGGAGTCTAA	GATTCAAATC	GAGGATCTAA	2640
	CAGAACTCGC	CGTGAAGACT	GGCGAACAGT	TCATACAGAG	TCTTTTACGA	CTCAATGACA	2700
	AGAAGAAAAT	CTTCGTCAAC	ATGGTGGAGC	ACGACACTCT	GGTCTACTCC	AAAAATGTCA	2760
15	AAGATACAGT	CTCAGAAGAC	CAAAGGGCTA	TTGAGACTTT	TCAACAAAGG	ATAATTTCCG	2820
	GAAACCTCCT	CGGATTCCAT	TGCCCAGCTA	TCTGTCACTT	CATCGAAAGG	ACAGTAGAAA	2880
20	AGGAAGGTGG	CTCCTACAAA	TGCCATCATT	GCGATAAAGG	AAAGGCTATC	ATTCAAGATG	2940
	CCTCTGCCGA	CAGTGGTCCC	AAAGATGGAC	CCCCACCCAC	GAGGAGCATC	GTGGAAAAAG	3000
	AAGACGTTCC	AACCACGTCT	TCAAAGCAAG	TGGATTGATG	TGACATCTCC	ACTGACGTAA	3060
25	GGGATGACGC	ACAATCCCAC	TATCCTTCGC	AAGACCCCTT	CTCTATATAA	GGAAGTTCAT	3120
	TTCATTTGGA	GAGGACACGC	TGAAATCACC	AGTCTCTCTC	TATAAATCTA	TCTCTCTCTC	3180
30	TATAACCATG	GACCCAGAAC	GACGCCCGGC	CGACATCCGC	CGTGCCACCG	AGGCGGACAT	3240
	GCCGGCGGTC	TGCACCATCG	TCAACCACTA	CATCGAGACA	AGCACGGTCA	ACTTCCGTAC	3300
	CGAGCCGCAG	GAACCGCAGG	AGTGGACGGA	CGACCTCGTC	CGTCTGCGGG	AGCGCTATCC	3360
35	CTGGCTCGTC	GCCGAGGTGG	ACGGCGAGGT	CGCCGGCATC	GCCTACGCGG	GCCCCTGGAA	3420
	GGCACGCAAC	GCCTACGACT	GGACGGCCGA	GTCGACCGTG	TACGTCTCCC	CCC GCCACCA	3480
40	GCGGACGGGA	CTGGGCTCCA	CGCTCTACAC	CCACCTGCTG	AAGTCCCTGG	AGGCACAGGG	3540
	CTTCAAGAGC	GTGGTCGCTG	TCATCGGGCT	GCCCAACGAC	CCGAGCGTGC	GCATGCACGA	3600
	GGCGCTCGGA	TATGCCCCCC	GCGGCATGCT	GCGGGCGGCC	GGCTTCAAGC	ACGGGAACTG	3660
45	GCATGACGTG	GGTTTCTGGC	AGCTGGACTT	CAGCCTGCCG	GTACCGCCCC	GTCCGGTCTT	3720
	GCCCGTCACC	GAGATCTGAT	CTCACGCGTC	TAGGATCCGA	AGCAGATCGT	TCAAACATTT	3780
50	GGCAATAAAG	TTTCTTAAGA	TTGAATCCTG	TTGCCGGTCT	TGCGATGATT	ATCATATAAT	3840
	TTCTGTTGAA	TTACGTTAAG	CATGTAATAA	TTAACATGTA	ATGCATGACG	TTATTTATGA	3900
55	GATGGGTTTT	TATGATTAGA	GTCCC GCAAT	TATACATTTA	ATACGCGATA	GAAAACAAAA	3960

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	TATAGCGCGC	AAACTAGGAT	AAATTATCGC	GCGCGGTGTC	ATCTATGTTA	CTAGATCGGG	4020
	AAGATCCTCT	AGAGTCGACC	TGCAGGCATG	CAAGCTTGGC	GTAATCATGG	TCATAGCTGT	4080
5	TTCCTGTGTG	AAATTGTTAT	CCGCTCACAA	TTCCACACAA	CATACGAGCC	GGAAGCATAA	4140
	AGTGTAAGC	CTGGGGTGCC	TAATGAGTGA	GCTAACTCAC	ATTAATTGCG	TTGCGCTCAC	4200
	TGCCCCGTTT	CCAGTCGGGA	AACCTGTCGT	GCCAGCTGCA	TTAATGAATC	GGCCAACGCG	4260
10	CGGGGAGAGG	CGGTTTGCGT	ATTGGGCGCT	CTTCCGCTTC	CTCGCTCACT	GACTCGCTGC	4320
	GCTCGGTCGT	TCGGCTGCGG	CGAGCGGTAT	CAGCTCACTC	AAAGGCGGTA	ATACGGTTAT	4380
15	CCACAGAATC	AGGGGATAAC	GCAGGAAAGA	ACATGTGAGC	AAAAGGCCAG	CAAAAGGCCA	4440
	GGAACCGTAA	AAAGGCCGCG	TTGCTGGCGT	TTTTCCATAG	GCTCCGCCCC	CCTGACGAGC	4500
	ATCACAAAAA	TCGACGCTCA	AGTCAGAGGT	GGCGAAACCC	GACAGGACTA	TAAAGATACC	4560
20	AGGCGTTTCC	CCCTGGAAGC	TCCCTCGTGC	GCTCTCCTGT	TCCGACCCTG	CCGCTTACCG	4620
	GATACCTGTC	CGCCTTTCTC	CCTTCGGGAA	GCGTGCGCT	TTCTCAATGC	TCACGCTGTA	4680
25	GGTATCTCAG	TTCGGTGTAG	GTCGTTGCT	CCAAGCTGGG	CTGTGTGCAC	GAACCCCCCG	4740
	TTCAGCCCGA	CCGCTGCGCC	TTATCCGGTA	ACTATCGTCT	TGAGTCCAAC	CCGGTAAGAC	4800
	ACGACTTATC	GCCACTGGCA	GCAGCCACTG	GTAACAGGAT	TAGCAGAGCG	AGGTATGTAG	4860
30	GCGGTGCTAC	AGAGTTCTTG	AAGTGGTGGC	CTAACTACGG	CTACACTAGA	AGGACAGTAT	4920
	TTGGTATCTG	CGCTCTGCTG	AAGCCAGTTA	CCTTCGGAAA	AAGAGTTGGT	AGCTCTTGAT	4980
35	CCGGCAAACA	AACCACCGCT	GGTAGCGGTG	GTTTTTTTGT	TTGCAAGCAG	CAGATTACGC	5040
	GCAGAAAAAA	AGGATCTCAA	GAAGATCCTT	TGATCTTTTC	TACGGGGTCT	GACGCTCAGT	5100
	GGAACGAAAA	CTCACGTTAA	GGGATTTTGG	TCATGAGATT	ATCAAAAAGG	ATCTTCACCT	5160
40	AGATCCTTTT	AAATTAAAAA	TGAAGTTTTA	AATCAATCTA	AAGTATATAT	GAGTAAACTT	5220
	GGTCTGACAG	TTACCAATGC	TTAATCAGTG	AGGCACCTAT	CTCAGCGATC	TGTCTATTTT	5280
45	GTTATCCAT	AGTTGCCTGA	CTCCCCGTG	TGTAGATAAC	TACGATACGG	GAGGGCTTAC	5340
	CATCTGGCCC	CAGTGCTGCA	ATGATACCGC	GAGACCCACG	CTCACC GGCT	CCAGATTTAT	5400
	CAGCAATAAA	CCAGCCAGCC	GGAAGGGCCG	AGCGCAGAAG	TGGTCCTGCA	ACTTTATCCG	5460
50	CCTCCATCCA	GTCTATTAAT	TGTTGCCGGG	AAGCTAGAGT	AAGTAGTTCG	CCAGTTAATA	5520
	GTTTGCGCAA	CGTTGTTGCC	ATTGCTACAG	GCATCGTGGT	GTCACGCTCG	TCGTTTGTA	5580
55	TGGCTTCATT	CAGCTCCGGT	TCCCAACGAT	CAAGGCGAGT	TACATGATCC	CCCATGTTGT	5640



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5 GCAAAAAAGC GGTTAGCTCC TTCGGTCCTC CGATCGTTGT CAGAAGTAAG TTGGCCGCAG 5700  
 TGTTCATCACT CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG CCATCCGTAA 5760  
 GATGCTTTTC TGTGACTGGT GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC 5820  
 GACCGAGTTG CTCTTGCCCG GCGTCAATAC GGGATAATAC CGCGCCACAT AGCAGAACTT 5880  
 10 TAAAAGTGCT CATCATTTGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG ATCTTACCGC 5940  
 TGTGAGATC CAGTTCGATG TAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA 6000  
 CTTTCACCAG CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA AAATGCCGCA AAAAAGGGAA 6060  
 15 TAAGGGCGAC ACGGAAATGT TGAATACTCA TACTCTTCCT TTTCAATAT TATTGAAGCA 6120  
 TTTATCAGGG TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC 6180  
 20 AAATAGGGGT TCCGCGCACA TTTCCCGGAA AAGTGCCACC TGACGTCTAA GAAACCATTA 6240  
 TTATCATGAC ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT C 6291

## (2) INFORMATION FOR SEQ ID NO: 5:

25 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 5560 base pairs  
 (B) TYPE: nucleic acid  
 30 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (iii) HYPOTHETICAL: NO  
 35 (iv) ANTI-SENSE: NO  
 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: T-DNA of plasmid pTHW142  
 40 (ix) FEATURE:  
 (A) NAME/KEY: -  
 (B) LOCATION: 1..25  
 45 (D) OTHER INFORMATION: /label= RB  
 /note= "right border sequence of octopine TL-DNA from  
 pTiB6S3"  
 (ix) FEATURE:  
 50 (A) NAME/KEY: -  
 (B) LOCATION: complement (84..296)  
 (D) OTHER INFORMATION: /label= 3'g7  
 /note= "3' untranslated region containing the  
 polyadenylation signal of gene 7 of Agrobacterium T-DNA"  
 55 (ix) FEATURE: